



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Our Ref: 1887-181 MIS/bh

Prior Application: 08/307,621

Examiner: L. Leary

Art Unit: 1211

April 10, 1997

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Dear Sir:

1. This is a request for filing under the file wrapper continuing application procedure, 37 C.F.R. 1.62, for a continuation of prior complete application Serial No. 08/307,621 filed March 23, 1992 under the title USE OF TRANSGLUTAMINASE INHIBITOR FOR THE TREATMENT OF SCAR TISSUE.

2. The name of the applicants as originally filed and current correspondence addresses are as follows:

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This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventors in this application are the same.

The inventorship for all the claims in this application is the same.

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3. The filing fee of \$385.00 made up as follows is to be charged to our Deposit Account No. 19-2253. A copy of our Deposit Account Order Form is enclosed:

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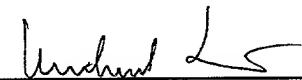
4. Please amend the specification by inserting before the first line the sentence:

"This is a continuation of copending application Serial No. 08/307,921 filed March 23, 1992."

5. The prior application is assigned to University of Manitoba under Reel 7201 Frame 0427.

6. The power of attorney in the prior application is to Peter W. McBurney, Reg. No. 19,352; Donald F. Sim, Q.C., Reg. No. 17,530; Michael I. Stewart, Reg. No. 24,973; Thomas T. Rieder, Reg. No. 22,862; Roger T. Hughes, Reg. No. 25,265; John H. Woodley, Reg. No. 27,093; Stephen J. Perry, Registration No. 32,107; Patricia A. Rae, Registration No. 33,570 and John R.S. Orange, Registration No. 29,725. The Power appears in the original papers in the prior application. Please address all future communications to: Sim & McBurney, 6th Floor, 330 University Avenue, Toronto, Ontario, Canada, M5G 1R7.

7. Please abandon the prior application upon withdrawal of the prior application from issue and at a time when this application is granted a filing date so as to make this application copending with said prior application.



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04/21/97

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Dear Sir:

PRELIMINARY REMARKS

In the parent application, Petition was submitted pursuant to 37 C.F.R. 1.313(b) (5) to withdraw this application from issue for the purpose of consideration of an Information Disclosure Statement under 37 C.F.R. 1.97 in this file wrapper continuation (FWC).

This FWC is accompanied by a PTO-1449 listing the Dissertation Thesis and Abstract thereof by one of the inventors and enclosing a copy thereof. It is not believed that this reference is relevant for the following reasons:

In considering this prior art, it is important to bear in mind the nature of the invention which is sought to be protected. The invention lies in the treatment of the occurrence of hypertrophic scar in humans using a non-toxic transglutaminase inhibitor. The formation of such scar tissue is itself an unpredictable event and is now known to arise from an accumulation of collagen type III in relation to type I.

The work done by the inventor on which the patent application is based was on human patients and it is believed that it was not predictable that the treatment described and claimed in the patent application would be successful in humans until such trials were conducted.

The Dolynchuk Thesis is a study of the mechanism of wound healing, with hypotheses being drawn from a prior study of the literature. It is stated on page 51:

"Review of the literature led to the hypothesis that glycoproteins other than collagen may be formed in wounded skin and that collagen-glycoprotein interactions may occur during wound healing."

"A second hypothesis was that transglutaminase may be involved in wound healing through glycoprotein cross-linking. This hypothesis arose from the findings of other workers which suggested that a role for transglutaminase in wound healing might involve isopeptide bond formation between fibrin and collagen, and from the finding in the present work (Section III, 1) that the [3^H] glucose-labelled material synthesized by wound tissue is difficult to solubilize."

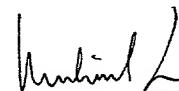
The final conclusion of the thesis (page 109) is:

"It is hypothesized that isopeptide cross-linking of the fucoprotein may be necessary for growth of collagen fibres in the wound tissue matrix. Therefore, use of transglutaminase inhibitors may be of therapeutic consideration in disorders of fibrous proliferation such as hypertrophic scar and other fibrocontractive disease processes."

This same paragraph is contained in the Thesis Abstract. The conclusion is a speculation as to what might be, based on observation in rats.

This paragraph does not, it is submitted, disclose or suggest the invention which is claimed. Having regard to the study conducted, it is a surprising result that hypertrophic scar tissue in humans may be treated and the results which have been obtained achieved.

These comments are submitted at this time to assist the Examiner in consideration of the relevance of the newly-cited art to the claimed subject matter.



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OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

		The Biochemistry of Wound Healing: The Characterization and Assembly of a Wound Fucoprotein by Kenneth N. Dolynchuk University of Manitoba 1986
		Abstract - Kenneth N. Dolynchuk
EXAMINER:		DATE CONSIDERED:

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 608; Draw line through citation if in conformance and not considered. Include copy of this form with next communication with applicant.

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THE BIOCHEMISTRY OF WOUND HEALING: THE CHARACTERIZATION AND ASSEMBLY OF A
WOUND FUCOPROTEIN

→ Author: DOLYANCHUK, RENNETH NICHOLIS

Degree: PH.D

Year: 1987

Corporate Source/Institution: THE UNIVERSITY OF MANITOBA (CANADA) (0303)
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Descriptors: CHEMISTRY, BIOCHEMISTRY

Descriptor Codes: 0487

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A review of the literature on the biochemical features of healing wounds suggested the hypothesis that glycoprotein biosynthesis might control collagen synthesis in patients with fibrocontractive disorders.

Full thickness wounds obtained from rats on various days post injury were incubated with $\text{Na}^3\text{sb2}\text{[sp135S]} \text{O}^3\text{sb4}$, plus either ($\text{S}^3\text{p3SH}$) fucose or ($\text{S}^3\text{p3SH}$) proline. The incorporation of label into various tissue fractions was compared with that found with unwounded skin. In agreement with previous reports, the maximum biosynthetic capacity for total collagen and proteoglycan occurred on day 5 post wounding. In contrast, peak incorporation of ($\text{S}^3\text{p3SH}$) fucose into urea/DTT extracted glycoproteins occurred on day 3 post injury. It was established by TCA or acid hydrolyses that ($\text{S}^3\text{p3SH}$) fucose was incorporated into glycoprotein oligosaccharide side chains.

50 mM putrescine, added to the incubation mixture to inhibit cross-linking by transglutaminase, was found to increase the fraction of the ($\text{S}^3\text{p3SH}$) fucoprotein which was soluble and to decrease the insoluble fraction. The substrate for the transglutaminase(s) in wound tissue were studied using ($\text{S}^3\text{p3SH}$) putrescine. This was found to label a 14 kilodalton protein. After exhaustive proteolytic digestion most of the protein was shown by HPLC to be in the form of γ -glutamyl ($\text{S}^3\text{p3SH}$) putrescine. The 14 kilodalton ($\text{S}^3\text{p3SH}$) putrescine-labelled protein co-migrated with a ($\text{S}^3\text{p3SH}$) fucoprotein on pore-gradient gel electrophoresis. This suggested that the fucoprotein contains a substrate site for transglutaminase.

The ($\text{S}^3\text{p3SH}$) fucoprotein in wound extracts made with urea/DTT showed major peaks on CL-6B chromatography at 42 and 14 kilodalton positions. Although the materials in the two peaks had similar amino-acid composition the 42 kilodalton fraction was not dissociated by boiling in 8M urea/50 mM DTT. In contrast, 42 kilodalton ($\text{S}^3\text{p3SH}$) putrescine-labelled material did not appear in urea/DTT extracts. This suggests that the 42 kilodalton fucoprotein may contain three 14 kilodalton subunits cross-linked by transglutaminase, and that its formation may be prevented by putrescine binding.

It is hypothesized that transglutaminase catalysed cross-linking of the fucoproteins is a necessary step in wound healing. Localized inhibition of this process may be of use in the treatment of disorders such as hypertrophic scars where collagen type III cross-linking is increased. (Abstract shortened with permission of author.)

THE BIOCHEMISTRY OF WOUND HEALING:
THE CHARACTERIZATION AND ASSEMBLY
OF A WOUND FUCOPROTEIN

BY

KENNETH R. DOLINCZUK

UNIVERSITY OF MASSACHUSETTS

THE BIOCHEMISTRY OF WOUND HEALING:
THE CHARACTERIZATION AND ASSEMBLY
OF
A WOUND FUCOPROTEIN

by

KENNETH N. DOLYNCHUK, M.D.

Submitted in defence of thesis for Ph.D.

1986

Department of Biochemistry, University of Manitoba,
Winnipeg, Manitoba,
Canada.

TO LUCY

"BY YOUR SCARS YOU WILL BE JUDGED." FITZ-GIBBON, BJPS, 1968.

"SURGICAL PERFECTION PER SE IS NOT THE COMPLETE ANSWER TO
OBTAINING FINE SCARS." Straatsma, PRS, 1947.

"THERE IS NO EVIDENCE AT ALL THAT GLYCOPROTEINS.... MAKE ANY
CONTRIBUTION TO TENSILE STRENGTH... THE EVIDENCE IS OVERWHELMING
THAT ALMOST ALL THE THE TENSILE STRENGTH OF THE WOUND IS DUE TO
COLLAGEN." PEACOCK, WOUND REPAIR, SECOND EDITION.

ACKNOWLEDGEMENTS

The author is indebted to the Medical Research Council of Canada, the Fire Fighters' Burn Fund, and the Department of Surgery, University of Manitoba, for their generous financial support.

Also appreciated was the technical assistance of Alan Tarr and Debbie Kellas. For their enthusiasm and friendly spirit when needed most, I will be forever grateful.

A special thanks is owing to Dr. M. Flint for his help in the final preparation of the thesis.

To Dr. J. M. Bowness, for his helpful insight and discussion, must go my deepest gratitude. His dedication and philosophy have been inspirational through the years and will continue to be so.

ABBREVIATIONS USED:

PMSF: phenylmethylsulfonylfluoride,

NEM: N-ethylmaleimide,

EDTA: ethylenediaminetetraacetate,

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid,

DTT: dithiothreitol,

SDS: sodium dodecyl sulfate,

DHEBA: N,N'-(1,2-dihydroxyethylene)bisacrylamide,

TBS: Tris buffered saline,

GAG : glycosaminoglycan ,

BBS: barbital buffered saline,

TCA: trichloroacetic acid,

TLC: thin layer chromatography,

HPLC: high performance liquid chromatography,

h: hours,

min: minutes,

sec: seconds,

c. p. m.: counts per minute,

d. p. m.: disintegrations per minute,

S. E.: standard error

fig(s): figure(s),

weights and measures : standard metric

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ABSTRACT

A review of the literature on the biochemical features of healing wounds suggested the hypothesis that glycoprotein biosynthesis might control collagen synthesis in patients with fibrocontractive disorders.

Full thickness wounds obtained from rats on various days post injury were incubated with $\text{Na}_2^{[35\text{S}]}O_4$, plus either $[^3\text{H}]$ fucose or $[^3\text{H}]$ proline. The incorporation of label into various tissue fractions was compared with that found with unwounded skin. In agreement with previous reports, the maximum biosynthetic capacity for total collagen and proteoglycan occurred on day 5 post wounding. In contrast, peak incorporation of $[^3\text{H}]$ fucose into urea / DTT extracted glycoproteins occurred on day 3 post injury. It was established by TLC of acid hydrolysates that $[^3\text{H}]$ fucose was incorporated into glycoprotein oligosaccharide side chains.

50 mM putrescine, added to the incubation mixture to inhibit cross-linking by transglutaminase, was found to increase the fraction of the $[^3\text{H}]$ fucoprotein which was soluble and to decrease the insoluble fraction. The substrate for the transglutaminase(s) in wound tissue were studied using $[^3\text{H}]$ putrescine. This was found to label a 14 kilodalton protein. After exhaustive proteolytic digestion most of the protein was shown by HPLC to be in the form of γ -glutamyl $[^3\text{H}]$ putrescine, the cross-linked product of transglutaminase action. The 14 kilodalton $[^3\text{H}]$ putrescine-labelled protein co-migrated with a $[^3\text{H}]$ fucoprotein on pore-gradient gel electrophoresis. This suggested that the fucoprotein contains a substrate site for transglutaminase. Subsequent findings by other workers, that the 15 kilodalton subunit of collagen type III aminopeptide is a fucoprotein and that this is an excellent substrate for transglutaminase, support this suggestion.

The $[^3\text{H}]$ fucoprotein in wound extracts made with urea / DTT showed major peaks on CL-6B chromatography at 42 and 14 kilodalton positions. Although the materials in the two peaks had similar amino-acid composition the 42 kilodalton fraction was not dissociated by boiling in 8M urea / 50 mM DTT. In contrast, 42 kilodalton $[^3\text{H}]$ putrescine-labelled material did not appear in urea / DTT extracts. This suggests that the 42 kilodalton fucoprotein may contain three 14 kilodalton subunits cross-linked by transglutaminase, and that its formation may be prevented by putrescine binding.

It is hypothesized that transglutaminase catalysed cross-linking of the fucoproteins is a necessary step in wound healing. Localized inhibition of this process may be of use in the treatment of disorders such as hypertrophic scar where collagen type III cross-linking is increased.

I. LITERATURE REVIEW

1. INTRODUCTORY REMARKS

The healing wound is very complex. To understand the clinical process of healing many important studies in the last half of this century have been directed at analysis of the structural components making up the wound matrix. Collagen biochemistry has been the most vigorously studied area of connective tissue research. As a result much is known of collagen structure, synthesis, chemical and structural interactions with itself and other matrix molecules⁵⁸⁻¹³⁶. However, we still have much to learn about the processes active in the healing wound which result in the orderly deposition and stabilization of collagen molecules into a structural array capable of withstanding increasing breaking forces after one week²⁶. This strength cannot be accounted for by fibrin clot which has begun to undergo dissolution by 72 hours post injury in the rat²³. Nor would it seem that collagen fibres of the order seen in photomicrographs of the early wound would be sufficient to maintain the structural integrity of the wound²⁷. Some suspect that structural molecules other than collagen may have a role in the orientation of the matrix and perhaps in the maintainence of tensile strength of early wounds^{7,8,9}. In contrast, there is considerable evidence that the organization of collagen proceeds *in vitro* without additional molecular or cellular components³³⁵⁻³³⁸. Nevertheless, clinical wound healing problems occur which are unexplained by current views. One particular clinical deficiency suggests that an

enzyme, transglutaminase, is involved in early wound healing³⁷⁵. It will be argued in this thesis that transglutaminase is responsible for cross-linking a fucoprotein which is present in wounds. When the enzyme is inhibited the extractability of this glycoprotein from the insoluble matrix increases *in vitro*. This wound fucoprotein shares certain structural and metabolic similarities with type III collagen aminopropeptide, and possibly is identical with it. It is proposed that the fucoprotein isolated is involved in formation of the wound matrix through intermolecular cross-linking by transglutaminase. In the predominantly type III collagen matrix this cross-link mechanism could possibly serve as a nucleation step in fibrillogenesis, for alignment of new fibrils, for arrangement of old with new collagen fibrils, or for limitation of fibril thickness. These possibilities will be made evident after presentation of the experimental data.

2. HISTORY OF WOUND HEALING

The study of wound healing has been an anachronism throughout the millenia owing to the slow acquisition of basic scientific understanding of the process. Prior to 1939, the clinical approach to the repair of tissues was observational and empirical, based on Halsted's¹ original principles, as well as those enunciated by Esmarch² in 1877. With the ensuing military experience, the phenomenological approach gave way to histochemical studies and an understanding that the active processes involved required a variety of cells in a changing environment. Knowledge of the histochemistry left many gaps in our understanding of tensile strength generation. Some of these were filled by the biochemical investigations of the fifties and sixties which led to an understanding of the basic principles of collagen fibre biosynthesis in the healing wound. The recognition that injured or deficient tissue matrix is replaced with newly synthesized fibrous protein allowed an opportunity to study turnover of collagen as well as other molecular constituents of the wound matrix. During the seventies what was known of collagen metabolism permitted the scientific approach to wound management including the development of artificial dermis, fibrin tissue sealant, hemostatic microfibrillar collagen, and cosmetic implantable collagen. Further appreciation of the features of cell-matrix interactions and chemotaxis in healing tissue with better control of events such as necrosis and inflammation has led to a decrease in the complication rate of surgery since Halsted's day.

In spite of numerous studies on the role of nutrition and tissue oxygenation, the

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conviction has grown that wounds heal at a maximal rate, which is limited by the capacity of normal healthy tissue in an optimum environment. Attempts to augment the reparative process beyond this normal capacity have met with minimal success. Reports of increasing tensile strength *in vivo* have been few, involving the use of autogenous blood, collagen powder, and dried fibronectin^{3,298}. Dunphy² has shown that secondary wounds heal at a faster rate than the primary wound, owing to the elimination of a discernable lag phase. This occurs only in the presence of preexistent wound matrix, and not if the primary wound is excised. Work by Peacock⁴, in which carbodiimide and methylene cross-links are introduced into collagen fibres *in vitro*, resulted in enhanced tensile strength of explanted wounds. Although this introduced the idea that collagen intermolecular cross-linking is an integral feature of structural repair, some unexplained observations remain intriguing. For example, the salutary effect of mercuric chloride on tensile strength generation prior to the production of collagen fibres in experimental wounds¹⁴. The production of disulfide bonds can be assumed to effect this result. Whereas, collagen types I and II have no intermolecular disulfide bonds, fibronectin and type III collagen chains do^{256,257,258,267}.

It is for the above reasons that study of the ground substance, which was conspicuous by its absence in the literature prior to 1953, has become an area of intense research. There are reports of associations between proteoglycan and collagen fibre formation, for example, as well as between fibrin, fibronectin and collagen^{5,6,10}. Glycoprotein has been implicated in the gain of tensile strength by various authors^{7,8,9}.

Although the precise nature and role of ground substance has remained elusive, substantial work over the past decade has demonstrated certain components with protean biological properties which are associated with cell-matrix interactions in soft connective tissues^{11,12}.

Because of the large number of papers on the subject this thesis will focus on the early phases of wound healing with particular reference to the role of glycoproteins. The prominence of fibronectin in the discussion is based on the important structural role it has been assigned in the connective tissue matrix²⁵⁹⁻²⁶⁴. However, the demonstration that laminin from basement membrane is a protein quite distinct from fibronectin^{265,266}, should emphasize the point that at present we do not know what fraction of the insoluble glycoproteins of any tissue are composed of fibronectin. In early incisional wounds a non-collagenous glycoprotein has been reported to be associated with fibronectin^{193,194}. In conjunction with collagen type III fibres the complex is known as reticulin. There is no clear biochemical evidence about the nature of this collagen-structural glycoprotein interaction. However, a tenable hypothesis would attempt to provide the underlying mechanism for complex formation. The rest of the literature survey will now be presented in order to support one hypothesis of how this may occur.

3. THE ULTRASTRUCTURE AND CLINICAL STAGES OF HEALING

The skins from various mammalian species differ in a few respects, some of which are characteristic for the species. However, the similarities allow for discussion of the processes essential for healing. The skin is a multilayered organ of mesenchymal and ectodermal origin. The relevance of the dermal-epidermal junction divides the stratum germinativum (the basal layer of the epidermis) from the underlying papillary dermis. While the cellular elements of the papillary dermis are engaged in activity related to scar formation, the proliferation of epidermal cells is well on the way to resurfacing the wound. In clean incisional wounds the latter process is complete within twenty-four hours, however, larger wounds require longer. The stages of epithelialization are: mobilization of the basal cells; migration; proliferation; differentiation. The last phase involves upward spreading of the basal cell derivatives in a continuum above the stratum germinativum. The stratum spinosum and the stratum germinativum, make up the Malpighian layer, with the stratum corneum above, which is distinguished from the Malpighian layer by being dead keratinized tissue. This subject is dealt with in detail elsewhere, but there is one further point concerning the division between the epidermis and dermis which is relevant to later discussion in this thesis¹⁴. Van der Shueren¹⁵ has recently examined the morphological characteristics in outgrowths of skin biopsies, and found that epidermal cells spread out onto the culture plate in advance of fibroblast activity. Epithelial cells remain polygonal, wrinkled, and closely opposed, without any basal lamina beneath migrating cells. Fibroblasts

maintain a smooth spindly shape, and attain the substratum beneath the epithelial cover. They accumulate microfilaments and microtubules in their cytoplasm, and have associated with their surface extracellular fibrillar material which resembles reticulin, or collagen. Others have shown that epidermal cells adhere to collagen type IV preferentially, and have found that it is a small sub population of round basal cells which adhere to the collagen substrata^{16,17}. It remains to be shown whether collagen of the basal lamina is produced by the underlying fibroblasts, as suggested by Abercrombie¹⁸, or is produced by the epithelial cell after some fibroblastic influence. Other basal lamina constituents, such as fibronectin are not contributed by the epidermis, whereas collagen type V is continually being produced during epiboly from the wound edge^{21,157}.

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REGENERATION VERSUS REPAIR : From the foregoing discussion it appears that the process of epithelialization has a time scale and a set of components which differ from those of other processes in the wound which provide it with uniquely important functions. Part of its integral role in the repair process is suggested by the fact that epithelial cells have collagenolytic activity ²². This enables the advancing edge of epidermal tissue to undermine the hemostatic clot and overlying dermis, causing this to slough²³. Once the scab has been removed at day three to four, before collagen synthesis is maximally under way, the epithelial covering provides, transiently, some tensile strength to the wound together with fibrin. Since the epidermis overlying the scar will never regain the thickness or durability of the original epithelium the repair process in skin is clearly distinct from that of regeneration . Thus skin, and other tissues of the body which form a scar, are different from liver, which has retained its ability to regenerate. The fact that epidermalization and scar formation are repair processes rather than regenerative ones is underscored by the phenomenon of contraction. Both the epidermal and dermal wound tissue possess contractile cells, containing actin-staining antigen ^{24,25} , which appear predominant during the early phases of cell migration and wound contraction in normal wounds. As pointed out later, the major ultrastructural feature of contractile cells, the actin plate or hemidesmosome, may have associated fibronectin on the external plasmalemma. Therefore, epithelialization and scar formation are repair processes which depend not only on the type of cell which is present, but also on the cell products in the intercellular matrix: the two are inexorably

related. The role of the fibroblast for example, depends on the type of intercellular protein it is producing. Nevertheless, early studies on inflammation were mainly concerned with the cell type present at various times after wounding. As our understanding of the biochemistry has improved the stages have become mutable, though still recognizably distinct. Therefore, the notion of a dynamic mosaic is more appropriate as a model, although the clinical stages remain entrenched in the literature.

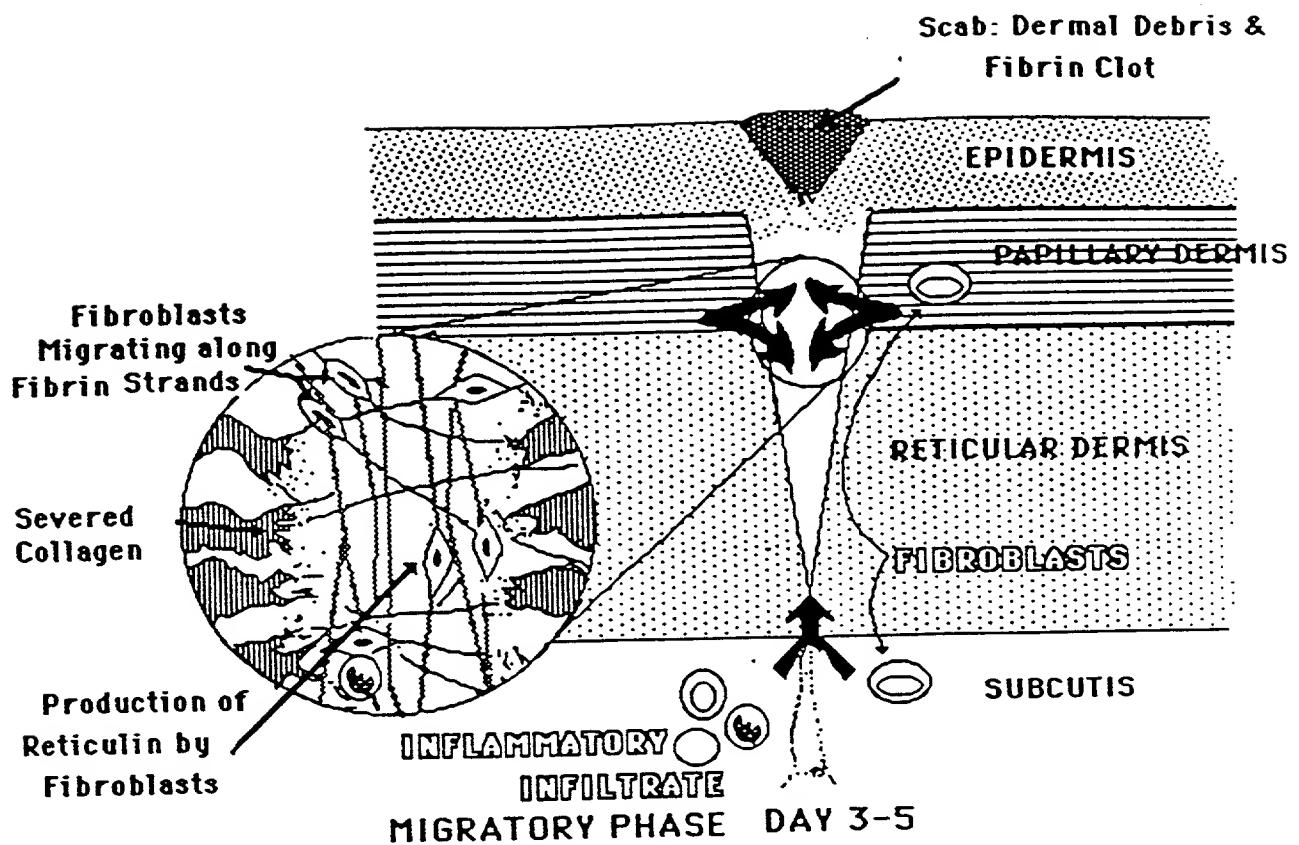
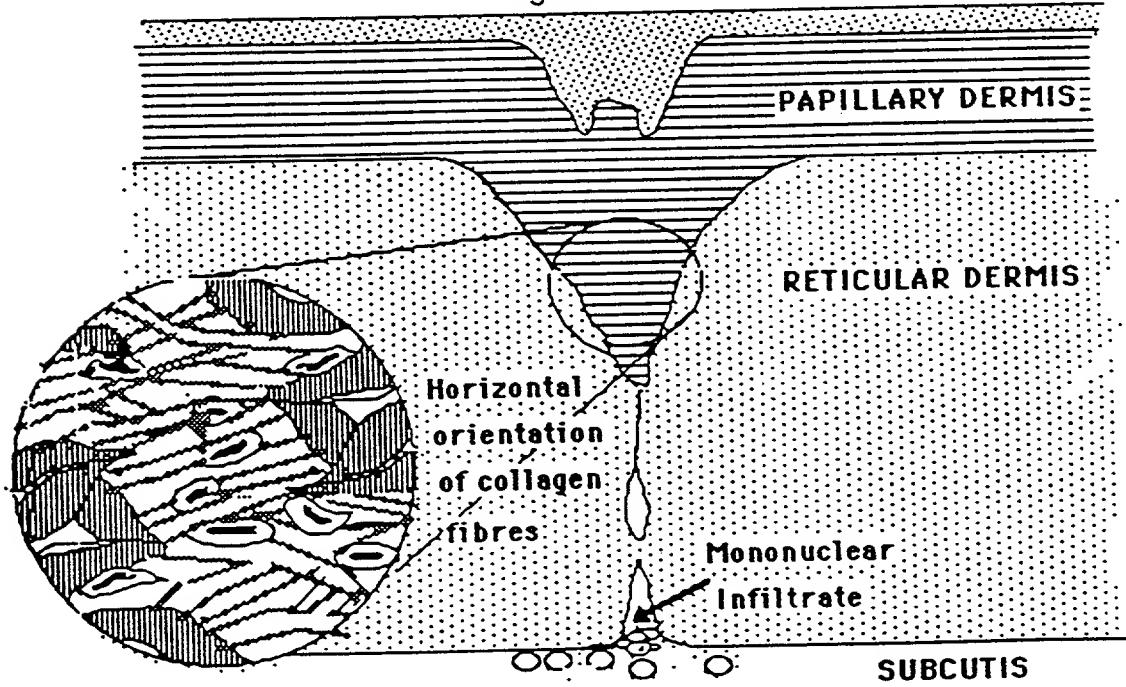


Figure 1.



REMODELLING PHASE DAY 7-21

Figure 2

14 CLINICAL STAGES OF WOUND HEALING ; NORMAL VERSUS PATHOLOGICAL

WOUNDS : The fundamental basis of wound repair in the intercellular matrix, as already intimated, is fibrogenesis. However, other important processes must occur in order for this to proceed normally. Generally speaking, hemostasis and migration / proliferation of cells constitute the inflammatory phase. When considering chronic granulation tissue, a stage of granuloma formation follows as well . Once the cellular activity in the wound is no longer increasing, a stage of remodelling occurs, followed by a prolonged period of maturation. Ordmann ²³ and Basset ²⁷ have provided the histochemical description of subepithelial wound healing superbly in their classic reviews. As summarized in figure 1, proliferation / migration is noted by the appearance of fibroblasts along fibrin strands with the production of collagen fibres along a vertical axis within the wound. Polymorphonuclear leukocytes and macrophage are seen entering the wound. During the subsequent remodelling period, as depicted in figure 2, the orientation of collagen becomes horizontal. Maturation continues for upwards of one year, but it is during the fourth to twelfth week that fibre diameter and packing density approach a maximum. This relates temporally to the rate of gain of tensile strength reaching a plateau at three months, as shown by Levenson²⁶. These and subsequent studies have been reviewed by Nicoletis and Delauney, and Boucek^{28,29,30,268}.

To summarize, during the first twenty four hours post-injury the production of a hemostatic plug, starting with platelet adhesion to collagen and other platelets, and followed by the events of blood coagulation, is the first reponse to injury. Increased

capillary permeability and diapedesis of red blood cells and active migration of polymorphonuclear leukocytes follow, secondary to humoral influences released by injured cells. Histamine is produced by tissue mast cells by decarboxylation of histidine. Serotonin is released from platelets and prostanoids are formed by the action of platelet phosphodioxygenase on lipid, made available from cell membranes, which are degraded by lipases released by the complement system. Other factors implicated are "lymph node permeability factor" and kinins, of which bradykinin is the archetype. The kinins are released from the α -2-globulins, kininogen I and II, by acid cathepsins and neutral proteases. Chemotaxis of polymorphonuclear leukocytes is mediated by prostanoid leukotrienes produced by degraded membrane phospholipids (*vide supra*). Other degraded matrix components, such as collagen, nucleic acid, histone protein, and fibronectin, which are produced by polymorphonuclear leukocyte cathepsins and neutral proteases act as chemotactic agents for other cells ^{271,272}.

The polymorphonuclear leukocytes are replaced by monocytid macrophage around the second day and these persist indefinitely. Their role in stimulating endothelial growth has been suggested by Greenberg³¹, and a purified angiogenesis factor has been characterized from wound chambers^{269,270}. Other factors have been described including transglutaminase in high concentration^{273,274}. Since endothelialization is complete by day seven, the major role of macrophage has been considered to be that of digesting dead polymorphonuclear leukocytes and cell matrix debris^{32,33,34}.

Eosinophils appear in wounds about day seven as well. Basset²⁷ has suggested

that their role in wound healing is concerned with remodelling of collagen. This is consistent with their timely appearance after inflammation, and with their possession of collagenase. Since severed collagen in the wound margin appears splayed at day three, it is likely that the polymorphonuclear leukocyte collagenase rather than the eosinophil enzyme is responsible at this early time. Furthermore, the type of collagenase produced by the polymorphonuclear leukocyte is specific for that type of collagen seen in the wound edge, as opposed to that type appearing in reticulin (*vide infra*)¹³⁰.

The cell which is largely responsible for production of the fibrous component of the wound as well as the ground substance is the fibroblast³⁵. Its origin is the mesenchymal adventitia surrounding blood vessels and the stroma of subcutaneous fat and epidermal appendages^{36,37,38}.

The origin of reticulin remains controversial. Basset²⁷ used refined silver staining techniques in order to confirm the existence of reticulin within the wound at twenty-four hours. This is consistent with Diegelman³⁹ and Cohen's⁴⁰ finding that collagen synthesis is beginning at this time. However, histologically defined collagen is demonstrable in fine interweaving bundles for the first time at day five. The predominant cells or "spindly basophilic cells with large nucleoli with vesicular nuclei indistinguishable from fibroblasts are prominent among the mitosing intralesional monocytes"²⁸. Grinnell⁴¹ has attributed this early argyrophilia to fibronectin, as discussed later with reference to the biochemical nature of structural glycoprotein in the

wound intercellular matrix.

Collagen fibres which are initially oriented perpendicular to the surface, become horizontally disposed by day ten where they remain associated with argyrophilic fibres until day fourteen²⁷. Upon lateral orientation the newly formed collagen can be seen to interdigitate with the existing dermal collagen ends, which have also shown marked argyrophilia since day two²⁷.

Remodelling is essentially complete by day twenty-one as the collagen fibres possess no argyrophilia and appear compact relative to earlier stages. Aside from enlargement of the collagen bundles and diminishing cellularity, no further histological changes occur during the maturation phase which may take up to a year and a half to complete²⁷.

Within granulating wounds much of the cellular activity seen in the dermis during the first six to seven days is similar to that of incisional wounds. Within twelve hours, wound exudate with polymorphonuclear leukocytes, red cells, macrophage, and fibrin is seen. The latter peaks at twenty-four hours and decreases between day three and four, while cells are maximally present at twenty-four hours and subsequently decrease after the third day. Cells resembling fibroblasts are definable at day six or seven, but collagen is stainable at day five as compared to incisional wounds. Leukocyte enzymes are prominent during the inflammatory phase. By nine days fibroblasts decrease and collagen increases in amount and becomes coarser. This description is the result of work by Russel, Ross, and Benditt⁴², on five millimeter punch biopsy wounds in guinea

pigs. Using larger wounds, Grillo and Watts⁴³ studied the process of contraction on the flanks of guinea pigs, and found that the area below the advancing edge contained numerous fibroblasts but little collagen as compared to the base of the wound. Contraction was largely an early process, being eighty percent complete by ten days. Drying of the scab was felt to be contributory during the first five days, but the rapid contraction from five to fifteen days was not explicable on this basis. Highton and James⁴⁴ showed that contraction forces on the wound edge were proportional to the amount of granulation tissue. Billingham and Medawar⁴⁵ previously showed that tension on the skin edges was accompanied by extensibility of the collagen of the skin itself; "intussusceptive" growth of the skin into wounds is a concomitant of wound contraction. The explanation of the phenomenon was elusive until it was realized that the myofibroblast, present in granulating wounds between day seven and twenty-one, is a contractile cell capable of generating tension through a distance of several centimeters on the order of 10^4 dynes cm^{-1} ^{25,46,47,275}. Most of what is currently known about myofibroblasts has been reviewed elsewhere⁴⁸.

It has already been mentioned that mobile fibroblasts contain contractile protein, and possess gap and tight junctions. These are reminiscent of the hemidesmosomes of pericytes, endothelial, and smooth muscle cells, with dense zones of fibrillar material between, which Singer⁵⁰ has shown to consist of fibronectin⁴⁹. Myofibroblasts possess fibronectin, but mature muscle cells do not^{52,53}. While normal fibroblasts and primitive mesenchymal cells express fibronectin, its expression in the latter appears

to be lost during differentiation to parenchymal derivatives⁵⁴. This would explain the presence of fibronectin in older wounds without invoking a source for it from plasma. Forrest⁹ has suggested that the myofibroblast, anchored to surrounding collagen by surface bound fibronectin, is the means by which early wound strength is maintained. The relationship of fibronectin to wound strength remains controversial, however (*vide infra*). The stimulus for conversion to more typical fibroblastic derivatives can only be speculated at, but it must occur during the period of maximal contraction, since the myofibroblasts disappear once wound closure is completed⁵⁵.

Montandon⁴⁹ has cited many of the factors which influence contraction, most of which act by either direct pharmacological inhibition of the contractile apparatus, or by interfering with myofibroblast proliferation. The latter is the most commonly used clinical modality, in the form of skin grafts and steroids. Grafting may act by blocking the ingress of fibroblasts from surrounding papillary dermis as well as effecting closure of the wound. More recent data on the level of prolyl-hydroxylase, the number of myofibroblasts beneath full-thickness grafts, and modulation of collagen type by split thickness skin grafts may help cast more light on the subject^{55,56,276}.

Pathological scars, fibromatoses, and fibrocontractive diseases have been studied in detail. For recent considerations referral to reviews by Nicholetis²⁸ and Chvapil²⁷⁷ is made. In concluding this section, however, Baur⁵⁵ has shown that typical myofibroblasts are present in the hypertrophic scar. It would appear that such a scar is abnormal in this respect, since active myofibroblastic features normally disappear once

the wound is closed. Resultant contracture or widening of the scar both denote varying degrees of hypertrophic activity^{28,57}. Non-specific histological changes may be predictive of hypertrophic scar formation²⁷⁸. However, it should be pointed out that unlike defective scars showing a whorled histological appearance, resulting from a preventable cause such as sepsis, inflammation need not be a late association to hypertrophic scars which have been placed across lines of relaxed skin tension. Keloid formation does not appear to fall precisely into this etiological classification, but controversy exists^{58,59,60}. The archetypal conditions of myofibroblastic hyperproliferation are the fibromatoses and fibrocontractive disorders. Treatment depends on excision of the involved nodular tissue, but recurrence is not prevented by such modalities. Radiation has been used, although no effect has been reported in those cases where the proliferation has already begun. Needless to say, were biochemical manipulation possible in patients with fibrocontractive disorders without attendant risk, this would be invaluable. Attempts at controlling collagen cross-linking have been attempted recently using β -amino-propionitrile²⁷⁹. However, the toxic effects of systemic use of this compound limit its clinical application. Subsequent studies on the effects of amino acid analogs on collagen accumulation has led to speculation as to their use in fibroproliferative disease^{349,367}. Specific control of collagen type III excess, which has been shown to occur in hypertrophic scar, has not been explored to date. This forms the basis for the proposed mechanism by which putrescine, and other transglutaminase inhibitors may act in preventing hypertrophic

scar, as discussed later in the experimental section of the thesis. (See also page 47.)

Some controversy exists as to the collagen composition of hypertrophic scar. Clore³¹¹ has suggested that type III collagen content is not increased using pepsin digestion and sieve chromatography. However, Weber⁴⁰⁸ has shown using cyanogen bromide cleavage and SDS electrophoresis that type III is increased and that the distribution of collagen peptides is almost that found in fetal skin.

4. BIOCHEMICAL COMPONENTS OF THE WOUND INTERCELLULAR MATRIX

COLLAGEN : Collagen, the major constituent of the connective tissue matrix has been studied in detail, and the subject has been reviewed thoroughly recently^{61,62,63}. A recent classification is included in Table I. The development of separation techniques specific for individual collagen types has relied on the physical-chemical properties, differential solubility in neutral salt solutions and dilute acid, susceptibility to proteolytic enzymes, and structural differences of the various collagens. Since these properties in turn define the criteria whereby collagen is distinguished from other structural proteins, they are outlined herein. Consideration of collagen self-interaction will be introduced in this section also.

Collagen is distinguished from other intercellular molecules by its relative resistance to cleavage by most proteases, its susceptibility to digestion by collagenase, and by the structural characteristic of the gly-X-Y triplet, where hydroxyproline occupies the the Y-

position in 10% of the amino acid sequence and hydroxylysine appears in variable amounts depending on the type of collagen. Some collagens are recognizable by their characteristic banding pattern of fibres in the electron microscope. It is possible, on the basis of these properties to differentiate collagens from C1q and acetylcholinesterase, which contain collagen-like sequences^{65,66}. In connective tissue, collagen accounts for about 20% of the total protein, and in repair tissue for greater than 50%⁶⁴.

Although there are at least nine characterized types of collagen known, which are the products of heterogeneous non-allelic loci, they have certain structural similarities. Further discussion will be limited to the interstitial collagens, type I-III, or class I, as they have the most bearing on subepithelial wound healing.

The collagen molecule consists of three type-specific chains in right-handed helical conformation, wound into a left-handed superhelix. The molecule, therefore, is a hybrid of either two similar chains with one dissimilar chain, or three identical chains. Type I, for example, can exist as either $[\alpha_1(I)]_3$ (Type I trimer), or $[\alpha_1(I)]_2 \alpha_2(I)$ ⁶⁷. Church and Tanzer⁶⁸ determined that precursor collagen, or procollagen, could be separated from collagen and further characterized as type I or III using gradient DEAE-cellulose after precipitation with ammonium sulfate, and ethanol precipitation, with 2 M urea in the eluting buffer. This technique found application to other types of procollagen owing to the inability of collagen to bind in the presence of urea to DEAE-cellulose^{68,69,70}. Extensive use of differential solubility of collagen in neutral salt and weak acetic acid

solutions, after pepsin and/or trypsin digestion has been used to separate type I from type III^{71,72}, chick bone collagen from type II⁷³, and type V^{75,76}. Tryggvason⁷⁷ using trypsin and pepsin reports that the two procollagen chains are found together with various smaller peptides. This earlier work suggested that peptides found in collagen fractionations may be the result of the extraction procedure. It was also shown in the latter study that the two procollagen chains are produced in the cell simultaneously. At the ribosomal level this would imply that chains destined to cross-link as procollagen molecules are present on adjacent ribosomes, and are processed from then on simultaneously. However, the intracellular and extracellular fate of procollagen molecules is still debated. Harwood⁷⁸ showed that type II procollagen chains like procollagen type I occurs on 250 S ribosomes, and that nascent chains can be processed by protohydroxylases, responsible for the synthesis of hydroxyproline and hydroxylysine. Sulfhydryl bonding between terminal propeptides occurs early after release into the rough endoplasmic reticulum, being complete by the time that the smooth endoplasmic reticulum is reached. That is, bonding and interchain cross-linking is responsible for allowing other post translational changes to occur to a greater extent for type II than for type I. This may also be true for type IV, which is known to possess more 3-hydroxyproline hydroxylysine and carbohydrate than any of the other types of collagen and which undergoes extensive intermolecular disulfide-bonding⁴¹⁰.

TABLE 1. COLLAGEN CLASSIFICATION-BURGESON¹⁷¹(1987)*

CLASS	TRIVIAL NAME	TYPE	CHAIN DESIGNATION
1	INTERSTITIAL COLLAGEN (MAJOR STRUCTURAL COLLAGENS)	I I trimer II major II minor III	[$\alpha_1(I)$] ₂ $\alpha_2(I)$ [$\alpha_1(I)$] ₃ [$\alpha_1(II\ M)$] ₃ [$\alpha_1(II\ m)$] ₃ [$\alpha_1(III)$] ₃
2	BASEMENT MEMBRANE COLLAGEN	IV	[$\alpha_1(IV)$] ₂ $\alpha_2(IV)$
3	PERICELLULAR COLLAGENS MINOR CARTILAGE COLLAGEN	V VI	[$\alpha_1(V)$] ₂ $\alpha_2(V)$ "AB2" [$\alpha_1(V)$] ₃ [$\alpha_3(V)$] ₃ $\alpha_1(V)$ $\alpha_2(V)$ $\alpha_3(V)$ [E] ₃ [F] ₃
4	DISCONTINUOUS TRIPLE HELICAL COLLAGENS	EC HMW, LMW, IX	[EC] ₃ [$\alpha_1(IX)$] ₃
	ACIDIC AND BASIC COLLAGEN FRAGMENTS (FUROTO & MILLER		DISULFIDE BONDED HIGH MOLECULAR WEIGHT AGGREGATES

* updated since publication in 1982 75-77, 99,104-119, 280, 398, 410.

TABLE 2 : BIOLOGICAL CHARACTERISTICS OF INTERSTITIAL COLLAGEN

TYPE	LOCATION	CHARACTERISTICS
COLLAGEN I	BONE, SKIN, TENDON, AND CORNEA	PRODUCT OF FIBRO-, OSTEOBLAST EPITHELIAL, AND SMOOTH MUSCLE CELLS LOW OH-LYSINE, LOW CARBOHYDRATE TYPE I TRIMER; MURINE BLASTOCYST, AND TERATOMA CULTURES ⁷⁹ ALSO IN DENTIN AND SKIN ⁸⁹
COLLAGEN II	CARTILAGE; LUNG BONE, VERTEBRAL DISC, SCLERA, AND BOWMAN'S CAPSULE	PRODUCED BY CHONDROCYTES, NEURAL RETINAL CELLS AND NOTOCORD CELLS. MORE POST-TRANSLATIONAL CHANGE THAN TYPE I ; 20 CARBOHYDRATE RESIDUES / CHAIN ⁹⁰⁻⁹⁵
COLLAGEN III	EYE LID ¹⁰⁰ ENDOTENDONIUM, ENDOMYSIUM, PERIMYSIUM, SUBENDOTHELIUM, NICTITATING MEMBRANE, IRIS, LIVER ^{95,97,98,99}	CODISTRIBUTES WITH TYPE I IN FIBROUS AND FETAL TISSUES ¹⁰¹ . PRESENT DURING EARLY WOUND REPAIR ^{102,103} . COMPONENT OF RETICULIN, CONFERS ELASTICITY ⁴⁸ CONTAINS CYSTEINE, LITTLE OH-LYS. PRODUCED BY FIBROBLASTS AND MYOFIBROBLASTS

The steps in the biosynthesis of collagen have been summarized above and reviewed elsewhere¹²⁰. Only post-translational modification will be considered in further detail, since this relates to the subsequent discussion. The modifications which first occur are the introduction of hydroxyl groups into certain proline and lysine residues. The location of these residues appears to be determined by the surrounding amino-acid sequence (*vide infra*), but the number of hydroxylations may also be restricted by triple helix formation. With type I α chains triple helix formation occurs relatively soon post translationally and this terminates the reactions. Prolyl-hydroxylases in the RER membrane catalyse the reaction in the presence of molecular oxygen and several cofactors. Required are ascorbic acid, α -ketoglutarate, and ferrous ion. Prolyl residues are either hydroxylated in the 3- or 4- position, depending on the preceding amino acid sequence. That is, glycyl residues usually precede 4-hydroxyproline. On the other hand, 3-hydroxylations appear where two proline residues occur in sequence, and the second proline is subsequently hydroxylated in the 4- position. Both proline and 4-hydroxyproline stabilize the triple helix, although no role has been ascribed to the 3-hydroxyl derivative. A separate enzyme is required for lysine hydroxylation, but similar cofactors appear necessary^{121,122}. Once hydroxylysine is formed further modifications can occur owing to the presence of specific glycosyltransferases, located in the smooth and rough endoplasmic reticulum of the cell. In type I glycosylation is restricted to a single hydroxylysine at position 87 in the α 1 chain, and to positions 87 and 174 in the α 2 chain^{123,124}.

Further modifications affect the terminal peptides of the procollagen molecule.

Separate enzymes, procollagen N-protease and procollagen C- protease remove the amino terminal and the carboxyl terminal propeptides of procollagen, respectively¹²⁰.

Specificity of these enzymes for the various collagen types exists, as shown by the N-terminal propeptides isolated from type I differ from those of type III³⁹². The latter are 100 daltons larger than those of type I and unlike other types possess interchain as well as intrachain disulfide bonds^{125,256}. Furthermore, type III aminopropeptides from dermatosparactic bovine skin have been found to possess fucosylated oligosaccharide units in N-asparaginyl linkage²⁸¹. Certainly, the liberated amino extension peptides show specificity in their negative feedback of collagen synthesis for type I and III¹²⁶.

As intimated above, the triple helix can form once the C-terminal propeptide has been translated, with interchain disulfide bonds therein providing the nucleus for subsequent interactions in the helical regions of the procollagen type II molecule⁴⁸. The rate constant for coil-helix transition of the aminopropeptide of procollagen type III is favourable for it being the nucleation site²⁵⁸, while the non-helical C-terminal propeptide probably confers solubility to procollagen type I and III, allowing it to be secreted by the cell. Once the C-terminal propeptide has been removed, the resultant precursor procollagen type I or III is capable of extracellular fibre formation¹²⁷. Small amounts of p-N-collagen are readily extractable from fetal bovine skin as the major form of type III procollagen precursor²⁵⁶. Fibre diameter is further controlled by the removal of the aminopropeptide in the maturing fibres by proaminopeptidase³⁹³.

Where p-N-collagen type III persists due to deficiency of aminopropeptidase, collagen fibres remain thin and immature as seen in dermatosparaxis. Presumably owing to its partially non-helical structure, the aminopropeptide causes steric interference with thick fibre formation. However, as a triple helical peptide of 45 kilodaltons, the aminopropeptide could occupy the "hole" in 4D staggered collagen fibrils exactly, supporting a registration role²⁵⁷. Similar mechanisms for fibre regulation have been suggested involving dermatan sulfate and fibronectin^{282, 230}.

Once the isolation of specific collagen types and the mechanism of intercellular processing of collagen was established, the characterization, tissue localization, and metabolism of each type could be approached. However the work is still incomplete.

Amino acid sequences are known for type I and type II and partially determined for type III^{63,394}. Analysis of the amino acid sequences reveal that each $\alpha 1$ chain shares greater homology across isotype class than with $\alpha 2$ chains of the same type, and that interspecies homology exists^{61,63}. Earlier studies attempted to explain collagen banding patterns on the basis of amino acid sequences known for type I^{80,81}. These findings suggest that hydrophobic interactions between fibrils are responsible for the D periodic symmetry of collagen, that unpaired positive charges contribute to fibrous long spacing collagen, and that small molecular interactions result in segment long spacing collagen patterns. Formation of collagen fibrils from solutions has been extensively studied with regard to understanding these interactions⁸²⁻⁸⁶. However, extension of

the fibrillogenic properties of native collagen *in vitro*, to the *in vivo* situation, exclusive of other matrix molecular interactions, seems incomplete in view of more recent data on procollagen metabolism presented above, and the singular lack of control mechanisms required for *in vitro* fibrillogenesis. This does not preclude the need for appropriate non-covalent interactions between growing collagen fibres in addition to covalent reactions between them.

Enzyme degradation of collagen by collagenase and various other proteases has received recent attention owing to the possible relationship to *in vivo* control mechanisms¹²⁸. Several aspects of this work have significance for the later discussion, and are presented here. Native collagen chains and fibrous collagen molecules are acted on by collagenase as an initial step in their turnover. Although, there appears to be remarkable conservation of a Gly-Leu or Gly-Ile area which is susceptible to cleavage, each collagen type may require its own specific collagenase^{131,132}. The cell types producing different collagenases may be specific as well. Since only one of many such Gly-leu or -Ile bonds are cleaved, a noncollagenous or "weak" portion of the helix has been envisioned^{61,116}. Other characteristics of this region are a gap area in the collagen fibril, and a Glu-Gal bound to a nearby hydroxylysine^{132,133}. That fibronectin is bound at this region as well is interesting in terms of its possible role in modulating collagenolytic activity *in vivo*¹³⁶. Collagen type III can also be cleaved by trypsin and thermolysin close to the collagenase cleavable site^{134,135}. The fact that collagen type III is more sensitive to endoprotease

attack than type I, suggests that this area is important for the rate limiting step in collagen degradation and its control. Procollagen precursor type III (p-N-collagen) has another important collagenase sensitive site giving rise to the Col 1-3 peptide, which is relatively resistant to collagenase owing to disulfide bonds within, and constitutes the whole amino-terminal precursor-specific region of bovine type III procollagen²⁵⁸. Therefore, collagenase may be important in the feedback regulation of collagen type III production (*vide supra*)¹²⁶.

PROTEOGLYCAN : The structure of cartilage proteoglycan has been described as a bottle-brush in excess of 10^6 daltons in size¹⁶⁹. Individual proteoglycan monomers consist of 50 or more glycosaminoglycan, henceforth abbreviated GAG, chains attached to a core protein. The proteoglycan monomers attach non-covalently to a single molecule of hyaluronic acid. This proteoglycan aggregate may be 100 -150 $\times 10^6$ daltons in size, and is stabilized by the third important component of link glycoprotein. There is some evidence that similar structures exist in skin. However, this has not been rigorously investigated.

GLYCOSAMINOGLYCAN : As proposed by Jeanloz¹³⁸ in 1960, the glycosaminoglycans are a class of molecules possessing a characteristic repeating disaccharide unit seen in all these polysaccharides except keratan sulfate which is actually a glycoprotein. They share many structural similarities and physical chemical properties yet display sufficient differences to be recognized as specific entities. Nomenclature remains essentially that as listed in table 3. The repeating unit consists

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of hexosamine, either glucosamine or galactosamine, alternating with a uronic acid, either glucuronic acid or L-iduronic acid. In keratan sulfate the hexosamine is glucosamine in type I or both glucosamine and galactosamine in type II, whereas, the uronic acid is replaced by galactose. The linear polysaccharide chains formed are linked to protein cores, which in distinction with most glycoproteins, are the minor components of the entire proteoglycan structure. Thus, heterogeneity among GAGs can

TABLE 3: GLYCOSAMINOGLYCANS OF CONNECTIVE TISSUE ¹⁴³

NAME	AMINO SUGAR	URONIC ACID	SULFATE	AMINO SUBSTITUTION	LINKAGE TO PROTEIN
HYALURONIC ACID	D-GlcN	D-GlcUA	-	N-AC	?
CHONDROITIN	D-GalN	D-GlcUA	-	N-AC	Gal-Gal-Xyl-Ser
CHONDROITIN-4-SO ₄	D-GalN	D-GlcUA	O-SO ₄	N-AC	Gal-Gal-Xyl-Ser
CHONDROITIN-6-SO ₄	D-GalN	D-GlcUA	O-SO ₄	N-AC	Gal-Gal-Xyl-Ser
DERMATAN SULFATE	D-GalN	L-IdUA D-GlcUA	O-SO ₄ O-SO ₄	N-AC	Gal-Gal-Xyl-Ser
HEPARAN SULFATE	D-GlcN	D-GlcUA L-IdUA	N-SO ₄ O-SO ₄	N-AC N-SO ₄	Gal-Gal-Xyl-Ser
HEPARIN	D-GlcN	D-GlcUA L-IdUA	N-SO ₄ O-SO ₄	N-SO ₄ (N-AC)	Gal-Gal-Xyl-Ser
KERATAN SULFATE I (CORNEA)	D-GlcN	Gal (mann)	O-SO ₄	N-AC	GlcNAc-AspNH ₂
KERATAN SULFATE II (SKELETAL)	D-GlcN D-GalN	Gal (mann)	O-SO ₄	N-AC	GalNAc-Ser GalNAc-Thr

be seen to be related to the chain composition, length, presence and degree of sulfation, and the mode of protein linkage. The types and structures of GAG have been reviewed elsewhere^{139,142,143,151}. High charge density is the result of numerous carboxyl groups and sulfate groups, or both, present in the carbohydrate portion of the proteoglycan moiety¹³⁹. The result is a large solvent domain and characteristic extracellular solute mobility effects¹⁴⁰. The polyanion effects have been used extensively for separation of GAG on ion exchange resins after enzymatic removal of the protein core and may also relate to the secondary and tertiary structure of the GAGs and proteoglycans¹⁴¹.

HYALURONIC ACID: Controversy exists as to whether hyaluronate is linked to protein. Hardingham has implicated it in aggregation of proteoglycan in cartilage^{152,153}. This is an extension of Hascall and Sajdera's¹⁴⁵ finding that "link glycoprotein" was possibly important for the aggregation of proteoglycan and could be found associated with GAG in dispersed form. Presumably link glycoprotein binds proteoglycan with hyaluronate for the purpose of complex stabilization^{154,156}. Lipid or protein bound intermediates in the formation of hyaluronate have not been clearly demonstrated¹⁴⁴. The amino sugar N-acetyl glucosamine is attached in β 1-4 linkage, and the uronide, D-glucuronic acid is linked β 1-3 without any sulfate esterification. Hyaluronic acid exists with chondroitin-4- and 6-sulfate in such organs as skin, tendon, and cell cultures of human diploid fibroblasts, in addition to dermatan sulfate¹⁴⁶. Its visco-elastic properties are evident where it predominates ie. in vitreous humor,

Wharton's jelly, and synovial fluid ¹⁴⁷.

CHONDROITIN AND CHONDROITIN SULFATES (CS): Chondroitin was first described in 1954, subsequent to the more highly sulfated forms chondroitin-4- and 6-sulfate ¹⁴⁸. Apart from the presence and location of the esterified sulfate and the galactosamine residues, all three forms share similarities of structure. All are linked to protein through xylosylserine linkage . They possess disaccharide units of N-acetylgalactosamine linked β 1-4 with glucuronic acid which is β 1-3 linked. The number of disaccharides per chain depends on the tissue of origin and species^{148,149,156}. The presence of hybrids is well recognized, in which galactosamine esterified at C-4 is present together with galactosamine esterified at C-6 in the same polysaccharide chain. A phylogenetic relationship exists for the relative proportions of each, as the ratio of 6- to 4-sulfate increases with age in cartilage ¹⁵⁰. In nucleus pulposis C-6-S as well as keratan sulfate increase with age ¹⁵⁸. Therefore, age and type of tissue determine the amount and type of sulfated GAG present. In wounds, alteration of GAG content and type is typically related to the stage of inflammation, as discussed later.

DERMATAN SULFATE (DS): DS differs from chondroitin-4- and 6- sulfate in that the major uronic acid is L-iduronic acid, not glucuronic acid. Some forms also contain some glucuronic acid, usually in copolymer blocks. Synthesis is similar to chondroitin sulfate except that 5-L-epimerase is able to invert D-glucuronic acid to form iduronate when a proximal sulfate is present. It is found in skin, sclera, scar tissue,

heart valves, umbilical cord, and walls of blood vessels. The skin form is sulfated at the 4-position. Glycosidic linkage to the core protein is through O-xylosylserine linkage, as in C-4-S and C-4-S. DS shows an age related decrease in content in skin and nucleus pulposus and concentration changes with maturation of tendon 159,284.

KERATAN SULFATE (KS): KS shares compositional similarities with N-asparagine linked glycoprotein. KS I, specific for cornea, and KS II, for skeletal tissue, do not contain uronic acid, but possess glucosamine. KS II has O-xylosylserine linkage, whereas KS I has N-glycosylamineasparaginyl linkage^{161,162,163}. KS is present with CS as part of the same proteoglycan in nucleus pulposus¹⁶³. In addition to repeating monosaccharides there often exist mannose, fucose, and sialic acid residues, together with N-acetylgalactosamine. Sulfation occurs at the 6-position of both monosaccharide residues.

HEPARIN (H) AND HEPARAN SULFATE (HS): Iduronic acid and, as with DS, glucuronic acid is present. Structure of the polysaccharide backbone is otherwise quite different and allows for the uniquely high levels of sulfation which are thought to be necessary for HS activity¹⁶⁶. The distribution of H and HS is quite different. Heparin being intracellular and HS being extracellular and membrane associated.

The biosynthesis and degradation of GAGs is beyond the scope of our discussion and has been reviewed by others^{142,168,169,170}.

GLYCOPROTEINS : As late as 1968 glycoprotein was regarded as having very little functional import in wound healing^{285,301}. Various examples of glycoproteins have been described since, which have been implicated in the organization of the dermal and granulation tissue matrices. The former have been reviewed elsewhere^{12,172,173}. As a class of molecules they are somewhat poorly characterized, aside from a few recently described components which will be discussed below. Because of the close association of non-collagenous glycoproteins with collagen the use of collagenase as well as chaotropic agents has been extensive in extraction procedures described 174-179. Robert¹⁸⁰ has proposed the use of the term "non-collagenous structural glycoprotein" in order to define the relative resistance of these glycoproteins to collagenase and stability to drastic extraction conditions. The effect of reduction has proved to be important in the improved isolation of the smaller subunits from aggregates seen previously.

Investigation of the inflammatory reaction in skin and subcutaneous tissue has led several investigators to examine wound tissue specifically. Since Houck *et al.*¹⁸⁴ described a parallel increase of non-collagenous protein, based on hexosamine content in croton oil induced wounds, other reports of wound glycoprotein have emerged¹⁸⁷⁻¹⁸⁹. Fishkin and Berenson¹⁸⁵ characterized a fucosylated glycoprotein from day 7 rat granulomata by ultracentrifugation and free boundary electrophoresis. Sedimentation coefficients and electrophoretic mobility corresponded to a molecular

weight range of 13-20 kilodaltons. They found water soluble components which differed from plasma glycoprotein. Extraction of collagenous material from wound tissue by different methods, employing enzymatic or acid treatment, resulted in immunohistological identification of a non-collagenous reticulin component (NCRC) by Pras *et al.* 396. Its association with collagen type III was assumed to reflect a molecular interaction with it. This glycoprotein component was shown to co-distribute as argyrophilic fibers with reticulin. In addition to NCRC, histologically defined reticulin has been shown to stain immunologically for fibronectin^{191,195,196}. Grinnell⁴¹ has provided evidence that fibronectin derived from plasma is the source of argyrophilia in reticulin. Fibronectin has been observed using immunohistochemical techniques to be present in wounds from rabbit, mouse, and guinea pig^{192,193,41}. However, localization of argyrophilia to areas associated with antifibronectin staining but not dependent on the presence of fibronectin itself, suggest that the presence of NCRC-collagen complex rather than fibronectin is important for the staining seen with silver. With the advent of cell culture techniques, study of extracellular matrix glycoproteins *in vitro* supported the above findings. In 1977, Sear *et al.*¹⁸¹ described the major fucosylated glycoproteins released into the medium of human skin fibroblast culture and incorporated into matrix. In addition to fibronectin, [³H] fucose was incorporated into three other major glycoprotein species of 45,000, 30,000, and 15,000 daltons when proteinase inhibitors were used. The samples were denatured with SDS, reduced, and alkylated before application on Bio-Gel A-5. Much is now known about fibronectin (*vide infra*). However, the lower molecular weight non-collagenous

glycoproteins were less well characterized. Since the nature of NCRC is unclear its relationship to these glycoproteins remains presumptive. The finding that NCRC can be solubilized by aqueous extraction suggests that it is a glycoprotein similar in nature to that isolated by Fishkin as 13,000-20,000 daltons, although the molecular weight of NCRC is unknown. It is possible then that the 15,000 dalton fucoprotein described by Sear is related, being found in soluble and insoluble forms. It may be speculated that a fucoprotein of ~ 15,000 daltons combines with fibronectin and collagen type III to form reticulin in wounds.

The relationship of glycoprotein with collagen in various tissues is an intimate

one, as originally suggested by Robert¹⁷³ using immunological studies with collagen antisera. Since that early work, various glycoproteins have been found in a similar intimate relationship with collagen from wounds of which the aminopropeptide of collagen type III and NCRC are the most recent^{395,396} (*vide infra*). Fibronectin is another which deserves separate mention.

The body of literature on fibronectin is large^{182,183,195,197, 250,252,263}.

Therefore, the biochemical characterization will not be reviewed here¹⁹⁸⁻²¹⁵.

Species and tissue specific differences exist in the carbohydrate portion of fibronectin¹⁹⁴. This has been described as occurring for collagen of the same type as well²¹⁶. In view of this heterogeneity it will be accepted that the term fibronectin refers to a family of structurally similar glycoproteins.

The distribution of fibronectin in normal mammalian tissues has been described

217-221, 223. As well, the intercellular and extracellular location of fibronectin using immunohistochemical techniques at the light and electron microscopic levels has been studied 222. It is not in intimate association with the cell surface, but is involved with distant cell-cell and cell-matrix interactions 50. Fibronectin is found to occupy the external position on cell membranes where intracellular actin filaments are coincident. In growing fibroblast culture systems numerous intracellular smooth muscle-like structures develop, which appear to attach to available surfaces and to aid in locomotion 295. At confluence these structures seem to disappear. If the culture is wounded by slicing it with a needle, the intracellular fibrillar system redevelops and cells migrate from the wound edge 46, 296. The ability of fibronectin to bind specifically to collagen, GAGs, and fibrin appear to aid in this process of cell locomotion, and in the maintainance of tissue integrity 227-267, 167, 292, 293. At least in combination with GAGs, fibronectin could provide for wound cohesion early in the reparative process 9. The demonstration that transglutaminase will cross-link fibronectin to collagen and fibrin *in vitro* has provided an additional possible mechanism for covalent interactions between structural components of the wound matrix 197, 291, 294. At this writing there has been no definitive evidence that such cross-linking takes place *in situ* after the initial formation of the fibrin clot 318. The isolation of isopeptide bonds from wound glycoproteins or collagen of the type shown in fig 3 would first be necessary in order to invoke transglutaminase activity 312.

Fibronectin binds denatured collagen and procollagen with greater affinity than

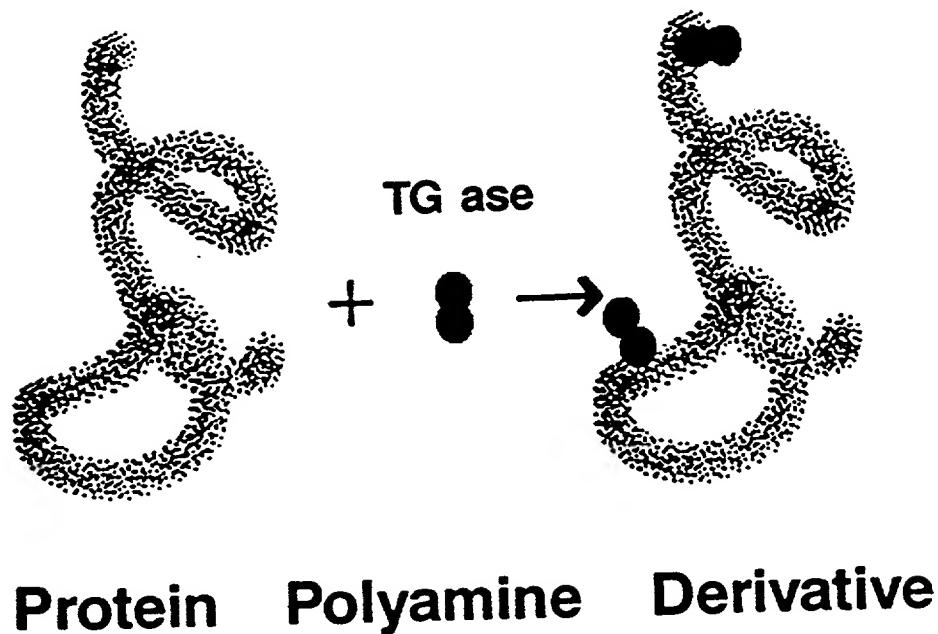
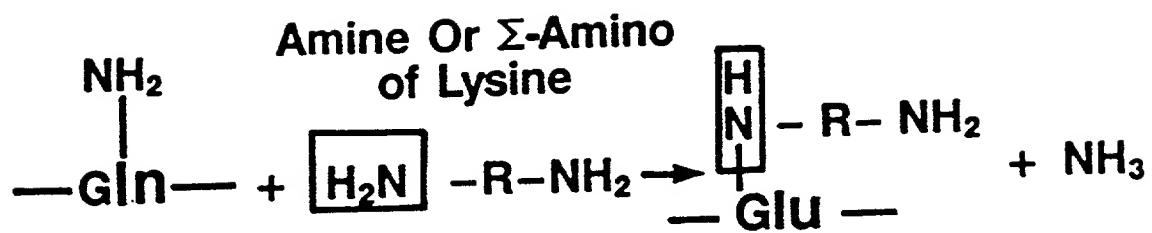
native forms and affinity to type III > I 252,284,313. As stated earlier, preexisting dermal collagen at the wound edge becomes splayed at day 3 under the light microscope, possibly providing sites for cell adhesion and collagen cross-linking (fig 1). Whether cell adhesion is necessary for new fibre formation to proceed across the wound can only be speculated at present 197,230,283. A scaffolding effect between surrounding dermal collagen, newly synthesized collagen, and fibronectin analogous to that suggested for organization of *in vitro* connective tissue matrices does seem attractive but unproven 9,263,300.

In conclusion, fibronectin has been shown to be intercalated with abundant collagen bundles by immunofluorescence, indicating some role for it in wound healing. Falconi *et al.* 298 has suggested that excess exogenous fibronectin increased the gain of breaking strength of incisional wounds by 30% after day 4. However, this has been suggested to be a non-specific effect of inflammation, and is contrary to the slower rate of fibrillogenesis seen in the presence of fibronectin *in vitro* 230,297.

Whereas fibronectin is associated with collagen type I and III, laminin and chondronectin are associated with types IV and II, possibly performing the analogous function of cell attachment for endodermal derivatives and chondrocytes repectively 250,251,253,290.

CHRONOLOGY OF SYNTHESIS AND TURNOVER IN THE MATRIX : Levenson 26 and Douglas³⁰⁷ showed that breaking strength increases over 90 days post-wounding, when the wound has only developed about 70% of its ultimate strength. This occurs in spite of decreasing collagen content after day 10³⁰¹. Once collagen content has stabilized after the second week, synthesis and breakdown

FIGURE 3 : ACTION OF TRANSGLUTAMINASE



remain in equilibrium; synthesis remains elevated over that of normal skin until day 70 29,308,319. The tight aggregation of collagen fibrils seen histologically at 100 days as compared to the open thin fibril architecture at 10 days would suggest that microarchitectural changes occur between 4-12 weeks. One suggestion is that the action of collagenase could compact collagen fibrils by removal of Col 1-3 peptides, without significantly affecting the collagen content (page 29)²⁵⁷. Based on the observation that collagen cross-links are constant in number and type during the 4-12 week period, others have proposed a role for glycoprotein in these processes ³⁰⁹. Attention, therefore, will be turned to the sequential appearance of wound matrix components in relation to collagen during the early phases of wound healing.

Type III collagen is found in proximity with myofibroblasts and in chronic granulation tissue. During normal healing a transition from type III to type I production is accompanied by an increase in the number of typical tissue fibroblasts. Although this relationship is suggested as a temporal one, Guber³⁰² states that it may be related to the fact that once plasticity of the wound is no longer required in bridging the defect, stability can ensue by such a transition. Collagen synthesis has been studied in granulating wounds by a number of authors with some controversy resulting. With the use of Cellstic® implants, Gay *et al.*¹⁰² found that in children, procollagen type III appeared 48 hours sooner than type I in the extracellular matrix. Immunofluorescence of type III was increased over that of type I until 96 hours, after which type I predominated. Clore *et al.*³¹⁰ disputed these findings owing to the presence of

foreign body reaction. Using pepsin / acetic acid extraction and the rat skin wound biopsy technique described by Diegelman³⁹ and Cohen⁴⁰ he found that type III was synthesized maximally at 10 hours, and that by 24 hours normal percentages were again found. It had previously been shown that type III contributes 20% to the collagen content of normal dermis³¹¹.

Using methods of extraction for cartilage GAGs applied to wounds, increases in chondroitin-4-sulfate and dermatan sulfate were noted at day 5 and increased until day 17³⁰⁶. However, as Carlsen *et al.*³⁰⁴ point out, by taking samples of tissue within 2.5 mm from the wound, this increase may be the peak of an earlier rise in one of the sulfated GAGs from the first 48 hours. This he found was followed by a later peak at 26 days and a final nadir at 50 days of what may be a different GAG type. As has been pointed out several times in the literature, the periphery of wounds is biochemically active^{40,303}.

Dunphy and Udupa³⁰⁵ found that the total hexosamine content of the wound was maximal at days 4-6. However, it was shown by Bentley¹⁸⁶ that 80% of this consisted of glycoprotein. Of the types of glycoprotein associated with skin wounds described earlier, only fibronectin has been studied sequentially. Kurkinen¹⁹³ first reported its presence in granulation tissue within 4 days of sponge implantation as reticulin fibres by silver impregnation. At 7 days fibronectin stained in all cellular areas. Procollagen III was not present at the advancing edge but was present behind it, using immunofluorescent staining. In contrast to data presented above in biopsy punch and

Cellstic® implant studies, collagen type I was seen in areas of type III procollagen staining. However, no procollagen type I was seen. At day 17 fibronectin and collagen were found in the same areas throughout the sponge with collagen appearing mature. At 3 weeks the sponge was filled with cells, more so toward the center. Distribution of fibronectin and collagen remained the same but staining was less intense for fibronectin as compared to procollagen III. At 5 weeks this was more marked. Grinnell 41 confirmed these findings in full-thickness granulating wounds. It was suggested that fibronectin is derived initially from plasma, in keeping with primary deficiency seen in post trauma patients during the first 48 hours³¹⁹. It is further suggested that when fibronectin stained in areas of collagen type I it was present with type III as well. By virtue of its ability to bind denatured type I more readily than the native molecule, fibronectin may contribute to the removal of type I by tissue macrophage. Repesh *et al.* 192 showed that in linear incisions fibronectin acted as a scaffold with fibrin for the migration of fibroblasts into the wound.

CLINICAL CORRELATES OF DEFICIENCY: No clinically defined congenital deficiencies of non-collagenous glycoprotein have been reported. The finding that transglutaminase deficiency leads to poor wound healing and spontaneous abortion has lead certain authors to speculate as to the importance of fibronectin cross-linking with fibrin and collagen in wound healing 320,321,41,192. Acquired fibronectin deficiency has been related to trauma and correlates with a decreased survivorship

due to sepsis 322. Although fibronectin deficiency has not been recognised in any wound healing disorder, one report suggests that an inherited platelet defect can be improved by treatment with fibronectin 299. Whatever the role of fibronectin it must be assumed to be basic to biological function, for no clinical correlate of an inherited deficiency has been described in humans to date. That is, a congenital deficiency of fibronectin is probably lethal. Deficiency of collagen type III has been described and classified as Ehlers Danlos Syndrome type IV 397. It is associated with pathologic scar formation which is unexpected, since hypertrophic scar is usually associated with increased amounts of type III relative to type I collagen 326. In some forms of osteogenesis imperfecta there is an imbalance of type III to type I collagen synthesis, although the effect may be limited to bone alone³²⁴.

5. BIOCHEMISTRY OF TENSILE STRENGTH GENERATION

COLLAGEN CROSS-LINKS ; EARLY VERSUS LATE : It has been the contention that collagen synthesis is a prerequisite to tensile strength of the wound once the fibrin clot has undergone dissolution 325. However, the mere presence of collagen in the extracellular wound matrix does not assure tissue integrity. Quantitative (*vide supra*) and qualitative changes in type I and III collagen can result in disturbance of the formation of intermolecular cross-links. The process can also be affected by a number of heritable and acquired disorders of lysyl oxidase function³²⁷.

The covalent cross-links which are generally accepted as occurring in the interstitial collagen of skin are basically of two types. Both types require aldehyde formation from the amine group of lysine or hydroxylysine. The first type is an aldol condensation reaction between allysine and another lysine or hydroxylysine, resulting in lysinonorleucine or hydroxylysinonorleucine, respectively. These are typically intramolecular cross-links between the telopeptides only. Thought to undergo reduction during maturation, the reduction products have not yet been isolated. The second pathway is based on hydroxyallysine which forms aldimines or Schiff base products with lysine or hydroxylysine, resulting in hydroxylysinonorleucine or dihydroxylysinonorleucine, respectively. The keto-form is spontaneously formed by Amadori rearrangement of the aldimine in both cases¹⁰³. The mature products are known to be lysyl pyridinoline and hydroxylysyl pyridinoline. The latter has been shown to be increased in hypertrophic scar and load bearing structures, but is absent in normal dermis, and mature scar³²⁸. It is known that the hydroxyallysine pathway is responsible for cross-linking early in wound healing with some pyridinoline formation possible, but that normally there is a changeover with time to the allysine pathway and its mature derivatives^{326,332}. It has been suggested that unusual cross-link combinations may result in the bizarre ultrastructural appearance of hypertrophic scar^{188,329,330}. An alternative suggestion is that persistence of embryological patterns of GAG may result in destabilizing effects on the development of the normal architecture of the scar^{28,147,331,333}.

There is recent evidence that basement membrane collagen network structure is maintained by reducible and non-reducible cross-links. The latter may be formed by transglutaminase^{398,410}.

OTHER FIBROUS COMPONENTS AND MECHANISMS OF FIBRILLOGENESIS :

Space does not permit a detailed discussion of the currently accepted models of *in vitro* collagen fibrillogenesis. Recent reviews have appeared which reveal that certain issues are still unclear^{335,336,337}. Whereas pure solutions of native collagen on heating will spontaneously undergo fibrillogenesis according to criteria specific for the conditions and type of collagen under study, it has not been determined how the process is initiated. There is electronmicrographic evidence supporting the formation of microfibrillar structures intracellularly, as segment-long-spacing collagen, which then aggregates in grooves on the surface of the cell^{337,339}. This microfibrillar initiation step has been disputed, however³³⁸. The subsequent assembly of intermediate sized aggregates then proceeds with influence from structural co-precipitants in the surrounding area. For example, proteoglycan has been shown to effect fibril diameter and rate of fibrillogenesis at this stage by interacting with fibrous-long-spacing collagen by binding to unpaired positive charges at either the N- or C-terminal of the collagen molecule^{6,80,343,344}. These charges are thought to affect linear growth, and contribute to the 4D stagger. Antiparallel dipole interactions would stabilize the 1D stagger, and are important for lateral aggregation. Recently, the 4D staggered dimer has been shown to be stabilized by both hydrophobic and ionic interactions brought

about by the β -fold conformation of the amino telopeptide predicted by amino acid analysis³⁴⁰. This arrangement brings hydroxylysine and lysine residues into approximation for covalent interactions as well.

Other experiments have been conducted studying the effects of proteoglycan and glycoprotein on fibrillogenesis. Anderson³⁴¹ showed that bovine tendon glycoprotein provided normal cross-striation patterns but thinner fibrils. The degree of fiber organization was shown to be dependent on the type of GAG-proteoglycan in discrete regions across the corneoscleral junction³⁴². Rajamaki and Kulonen¹⁹⁰ isolated a glycoprotein from rat granulation tissue which was extractable by collagenase and which increased collagen fiber formation. In examining the rate of fibril formation in the presence of bovine nasal proteoglycan, Lowther and Natarajan³⁴³ found that unaggregated proteoglycan inhibited collagen fibre formation. Addition of glycoprotein caused aggregation of the proteoglycan and subsequent disinhibition. The nature of these saline extractable glycoproteins is heterogeneous. However, the finding that fibronectin inhibited fibrillogenesis in a concentration dependent manner has assigned a possible role to this glycoprotein in collagen fibre organization²³⁰. Although fibronectin has yet to be isolated from scar collagen biochemically, it is present immunohistologically as stated earlier. In the wound it may quickly be modified by protease attack³⁹³.

The intimate association of glycoprotein with guinea pig scar collagen was reported by Forrest and Jackson¹⁸⁸, although the characterization was incomplete. It

was suggested that the glycoprotein provided thermal stability and dilute acid solubility characteristic of young embryonic collagen. In further work on the intermolecular cross-link differences between dermal and scar collagen, elevated dihydroxylysinonorleucine content in the latter was suggested as an explanation for the early physico-chemical properties seen³³².

CLINICAL CORRELATES OF ABNORMAL CROSS-LINKING: From the foregoing discussion it would seem possible to have abnormal cross-link formation on the basis of defective or deficient lysyl oxidase. This deficiency has been described in humans as Ehlers-Danlos syndrome type V and in the skin of some patients with cutis laxa³²³. Lysine hydroxylase is deficient in EDS type VI with resulting weak cross-linking between collagen molecules. The consequences are scoliosis and hyperextensible skin and ligaments³⁴⁸. Dermatosparaxis is another enzymatic deficiency state seen in cattle and sheep. The inability to cleave the aminopropeptide from procollagen by aminopropeptidase, which is deficient, results in thin skin and poor wound healing. EDS VII is a less severe but similar condition in humans caused by structurally abnormal collagen. This cannot be cleaved by the normally functioning aminopropeptidase³⁴⁸. Acquired or secondary forms of abnormal collagen cross-linking result from ingestion of nitriles, and therapeutic use of D-penicillamine. The latter resembles homocysteine and interferes with cross-linking by binding to hydroxylysine residues^{390,391}. The use of other amino acid analogues has been studied as to their regulatory effects on collagen³⁴⁹. The conversion of procollagen type II to collagen by cleavage of the C-terminal propeptide is reported to be inhibited by hexanoic acid and polyamines with resulting accumulation of procollagen³⁶⁷.

II. HYPOTHESIS AND RATIONALE

Review of the literature led to the hypothesis that glycoproteins other than collagen may be formed in wounded skin and that collagen-glycoprotein interactions may occur during wound healing. To test the first part of this hypothesis an experimental comparison of the biosynthesis of fucosylated glycoprotein and collagen by wounded skin explants *in vitro* was planned (Section III, 1). The rationale for the use of [³H] fucose as a label for glycoprotein was firstly that this sugar is not converted to other compounds readily and secondly that it does not occur in collagen. The subsequent report that the aminopropeptide of collagen type III is a fucosylated glycoprotein by Shinkai and Lapierre ²⁸¹ modified the interpretation of these results (see General Discussion). TLC experiments were planned to confirm that the label incorporated into glycoprotein was indeed [³H] fucose (Section III, 2).

A second hypothesis was that transglutaminase may be involved in wound healing through glycoprotein cross-linking. This hypothesis arose from the findings of other workers which suggested that a role for transglutaminase in wound healing might involve isopeptide bond formation between fibrin and collagen, and from the finding in the present work (Section III, 1) that the [³H] fucose-labelled material synthesized by wound tissue is difficult to solubilize. This second hypothesis was tested by the use of putrescine, a transglutaminase inhibitor, and [³H] putrescine, a probe of transglutaminase substrates in the healing wound (Section III, 4). Finally, efforts were made to characterize the proteins which labelled with [³H] fucose and [³H] putrescine.

III EXPERIMENTAL

1. THE BIOSYNTHESIS OF STRUCTURAL COMPONENTS IN WOUNDS

INTRODUCTION : It is known that different classes of glycoproteins exist in biological tissues. Recent reviews have appeared on the subject and are summarized here^{406,407}.

Berger *et al.* 406 classify glycoproteins into two major types, O-glycosidic and N-glycosidic. The main O-glycosidic groups are the mucin type of glycoproteins, characterized by sugar chains being attached to a polypeptide backbone by O-glycosidic linkage from N-acetylgalactosamine to serine or threonine. However, the collagens, which contain glucosyl galactosyl hydroxylysine and proteoglycans which contain glycosaminoglycan chains linked to serine or threonine through a sugar such as xylose represent two other groups of O-glycosidic protein-carbohydrate conjugates. The second major type are the N-asparagine-linked glycoproteins characteristic of cell-surface and intracellular as well as extracellular glycoproteins. This type is subclassified as being high mannose ("simple") or "complex" type. The carbohydrate-protein linking is through N-glycosidic bonds from N-acetylglucosamine to the amide of asparagine. Analysis of the mucin type has relied on reduction and methylation followed by acid hydrolysis of the carbohydrate entity. Use of N-glycosidases has benefitted the analysis of asparagine-linked glycoproteins. The N-acetylglucosamine or fucosyl-N-acetylglucosamine can be split off by chemical

cleavage using hydrazinolysis. There is much microheterogeneity of both the high mannose and complex types of glycoproteins in terms of their branching patterns. The presence of dolichol phosphate as the intermediate in the production of both these types is the common initiation step. After production of the core N-acetylchitobiose, glycosyltransferases along the RER are responsible for the addition of carbohydrate moieties during biosynthesis of the individual glycoproteins in an ordered sequence. Branching is dependent on the ordinate position of specific residues. The control of the resulting sequence is thought to be due to four governing factors: (1) competition for a given substrate, (2) substrate specificity for the transferase, (3) substrate availability , and (4) other factors such as pH, cations, and phosphate concentration⁴⁰⁶.

In mucin type glycoprotein fucose is present as a terminal sugar either attached to the penultimate galactose or N-acetylgalactosamine, blood group substances being the exception. In N-glycosidic glycoproteins fucose is present either attached to the terminal galactose or to the core N-acetylglucosamine. Fucosyltransferases are present in the RER to provide the final transferase reaction before transport out into the Golgi apparatus. Based on the foregoing information it was necessary to assure that adequate time for incorporation of fucose was allowed during incubations carried out with explanted wound tissue. The published literature supports the choice of a 6 hour incubation for maximal incorporation into fucosylated glycoproteins³⁹⁹. Since fucose is normally a product of mannose metabolism, and is not converted to other sugars and

excess fucose is transported out of the cell readily³⁹⁹, concentrations of [³H] fucose chosen for the incorporation into matrix glycoprotein were set arbitrarily in order to achieve sufficient radioactive recovery in later fractionations. The glycoprotein labelled was assumed to be N-asparaginyl in type. However, further confirmation of this was beyond the scope of our limited objectives. As well, measurement of pool sizes would require a different analytical approach to be used.

Owing to the disadvantages of foreign body implantation, the rat skin wound biopsy model was chosen because of its reproducibility^{39,40}. This technique was utilized to establish whether wound tissue in culture synthesizes fucosylated glycoprotein. The principal objective of this phase of the work was to determine whether wounded skin develops the capacity to produce fucosylated glycoprotein before, during, or after the period of maximum collagen and glycosaminoglycan synthetic capacity. [³H] fucose was originally chosen as a label for glycoprotein because of the demonstrated incorporation into fibroblast glycoproteins and fibronectin¹⁸¹. Subsequently it was shown to be present in the aminopropeptide of procollagen type III²⁸¹. The use of [³H] proline, followed by collagenase digestion, has been used reliably and widely to measure incorporation into newly synthesized collagen^{39,40}. Na₂[³⁵S]O₄ is a commonly used label for sulfated GAG synthesis⁴⁰⁰.

MATERIALS AND METHODS :

SURGICAL WOUNDING OF ANIMALS: Thirty-two male Sprague-Dawley

rats (100-130 g) were anaesthetized with Nembutal® (50 mg/kg intraperitoneally) and their backs were then closely shaved and swabbed with 70% ethanol. Wounds were created under semi-aseptic technique with a 3 mm dermal biopsy punch. All wounds were full thickness, through panniculus carnosus, as described by Cohen *et al.*⁴⁰. The individual wounds were 1 cm distant from each other, on the dorsi of the rats, such that there were 5 wounds on one side of the midline and 5 on the other. The wounds were dressed with Op Site® after the procedure for one animal was complete. The animals were killed with chloroform at 1,2,3,5,7,11,15, and 21 days after wounding and the dorsal hide removed *en bloc* at the plane of easiest dissection. Two animals were sacrificed at each time for a single set of determinations. The areas of granulation were measured with calipers and then harvested with a 4 mm dermal biopsy punch. Normal, unwounded skin was similarly harvested from the four corners of the resected hide and used as the controls of that animal.

INCUBATION OF TISSUE EXPLANTS: Approximately 100 mg of wound or normal skin was placed in 20 ml incubation flasks containing five 4 mm wound tissue discs or four 6 mm skin tissue discs. These tissue explants were then incubated in 3 ml of Krebs-Ringer bicarbonate buffered media modified after Uitto³⁵⁰, and Cohen *et al.*⁴⁰. 10 µl of [³H] proline (10 µCi) or 50 µl of [³H] fucose (50 µCi) were added to the tissues of one or the other animal of the pair. 50 µl of Na₂[³⁵S]O₄ (78 µCi) was then added to each flask and all were incubated for two hours at 37° under 5% CO₂ and

95% O₂ in a shaking water bath. Incubation was stopped by freezing to 0° and the flasks were stored at this temperature until used.

ASSAY OF LABELLED MATERIAL; The incubation mixtures were thawed and the tissue discs removed and placed in 6 ml of buffer containing 0.154 M NaCl, 15% glycerol (V/V), 50 mM Tris/HCl, pH 6.8, with 2 mM phenylmethylsulfonylflouride (PMSF) and 1 mM N-ethylmaleimide (NEM). The buffer for the assay of [³H] fucose in structural glycoprotein contained 2 mM EDTA as well. Samples were homogenized for 60 sec and for a further 30 sec after sharply mincing with scissor any remaining large skin fragments at 3/4 speed with a Polytron® homogenizer (Brinkman Instrument Co.) at 4° . The homogenate was then boiled for 10 min (fungicidal), dialysed against distilled water and the retentate lyophilized. Freeze-dried samples were weighed and divided into two by weight; equal portions (5-10 mg) were then assayed for either [³⁵S] or [³H] as described below.

[³H] PROLINE IN COLLAGEN AND COLLAGENASE RESISTANT RESIDUE; The methods of Peterkowsky and Diegelmann³⁵¹, and Cohen *et al.* ⁴⁰, were used as follows. The lyophilized material was extracted twice with 5% TCA , followed by extraction with ethanol : ether (3 : 1 by volume), removal of the solvent and evaporation to dryness overnight. The residue was suspended in 1 ml of 0.2 N NaOH and shaken at 37° for 1 h. Sonication for 15 sec produced a well-dispersed suspension which was then partially neutralized with 80 µl of 1 N HCl and mixed with

0.82 ml of buffer containing 500 μ mole HEPES (pH 7.2), 1.25 μ mole CaCl_2 and 6.25 μ mole NEM. To the 1.9 ml of buffered suspension was added 90 μ l of 0.05 M Tris/HCl, containing 5 mM CaCl_2 , and 10 μ l of collagenase (Advanced Biofactors Form III, 30 units/ μ l). The mixture (final pH. 7.3) was then incubated for 24 h at 37°. Digestion was stopped by addition of 0.25 ml of 50% TCA and 0.25 ml of 50% tannic acid. The resultant mixture was centrifuged at 15,000 \times g for 10 min and the supernatant filtered through a 0.8 μ Millipore® filter. 1.0 ml of the filtrate was taken for counting with 10 ml of ACS scintillant (Amersham). The insoluble residue was resuspended in 1.0 ml of 0.2 N NaOH overnight at 20° and counted in 10 ml of ACS scintillant.

[^{35}S] GLYCOSAMINOGLYCAN ASSAY; The procedure of Calatroni *et al.* 352, as described by Barry and Bowness 353 was modified as follows. In the original weighed freeze-drying flasks samples were suspended in 5 ml of 0.154 M NaCL in 0.04 M sodium barbital buffer (pH 8.0) and sonicated for 15 sec to disperse the particles finely. 0.4 ml of 0.5 M cysteine HCl and 0.4 ml of 0.18 M EDTA (tetrasodium) was added and the pH adjusted to 7.1 with HCl. 0.2 ml of papain (5 mg of Sigma 2X crystallized in suspension) was added and the mixture incubated for 24 h at 57°. The digest was then centrifuged at 31,000 \times g for 20 min. The supernatant was removed and the pellet washed with 1 ml of 0.154 M NaCl and the washings added to the original supernatant, giving a final volume of 6.8 ml. 0.95 ml of distilled water was added to bring the ionic strength to 0.154 and then 0.3 g of ECTEOLA cellulose,

activated as described by Barry and Bowness ³⁵³ was mixed with the solution on a rotator at 20° overnight. The ECTEOLA cellulose was then pelleted by centrifuging at 31,000 X g for 20 min and washed twice by resuspension in 10 ml of 0.154 M NaCL and centrifuging. Counting of some supernatants and washes showed that adsorption of [³⁵ S]- GAG was nearly complete. Desorption of glycosaminoglycans was carried out with 2.0 ml of 4 M NaCl at 4° for 48 h on a rotator. After centrifuging at 31,000 X g for 20 min the supernatant was drawn off and filtered as above. 1 ml of the desorbate (in 2.2 M NaCL) was diluted with water to give a concentration of 0.9 M NaCl. 1.5 ml of this solution was mixed with 10 ml of ACS and scintillation counting carried out.

[³H] FUCOSE IN GLYCOPROTEIN ; Weighed freeze-dried tissue homogenates were extracted with 3 ml of 1.65 M NaCl in tared 7 ml screw cap centrifuge tubes for 24 h on a rotatator at 4°. The mixture was centrifuged at 31,000 X g for 20 min and the supernatant removed as fully as possible. 2.0 ml of an 8 M urea, 50 mM dithiothreitol (DTT) solution was made in the tube by adding distilled water and weighed amounts of the solid reagents to the weighed amount of water remaining in the tubes. The mixture was sonicated for 15 sec and extracted for 48 h at 20° on a rotator. The mixture was centrifuged at 10,000 X g for 30 min and 0.5 ml of the supernatant was mixed with 10 ml of ACS and counted. The insoluble residues from the urea/DTT extraction were mixed with 1 ml N NaOH and counted with ACS.

RESULTS: In studying the metabolism of human skin samples, Utto ⁴⁰¹ recommended that slices 0.5 mm thick or less be made with a Stadie-Riggs microtome.

The rat skin sample discs used in the present study were 1.0 -1.5 mm thick, and it was found by experiment that 0.5 mm thick slices made from discs using the Stadie-Riggs dermatome incorporated less of the labelled compounds than did the intact discs. It is considered likely that the decreased incorporation was due to tissue damage from the pressure required to make slices from these small pieces of tissue. For these reasons all the experiments reported were done with intact skin discs.

The mean test / control ratios in figs 4 and 5 show the sequential changes in biosynthetic capacity of the wound tissue discs for incorporation of the labelled precursors into collagen, sulfated glycosaminoglycans, and fucosylated glycoprotein during the period 1-21 days post wounding. Since wound discs were always incubated at the same time as control discs obtained from the same animal on the same day the ratios eliminate some of the day to day variation in efficiency of the *in vitro* incubation technique which were observed during the course of the experiments. That the peaks shown in figs 4 and 5 are significant is shown by comparison of actual counts and derived ratios for the control and wound discs at various times (table 4a). The need to pool data for table 4 arose because comparison of data from individual days did not achieve statistical significance. It was reasonable to pool days 1-5 and 7-21 since the data reflect a general decrease in relative incorporation into the three types of structural components studied after day 5 (see table 4a). Comparison of specific activities for [³H] fucose in the urea /DTT extracts for pooled data from days 1-5 (see table 4) shows that there is less than a 1% chance that the tests belong to the same

TABLE 4. Specific activities of various tissue fractions, expressed as d.p.m. per mg of freeze-dried, dialysed, tissue homogenate.

Means \pm S.E. (with number of replicates in brackets)

Tissue fraction and labelling precursor	Control skin plugs	Wound plugs	P for 't' test of no difference
[³ H] fucose in urea/DTT			
Days 1-5	794 \pm 260 (8)	3420 \pm 573 (16)	<0.01
Days 7-21	937 \pm 295 (8)	1681 \pm 225 (15)	0.07
Days 1-21 (controls) vs. Days 1-3 (tests)	865 \pm 191 (16)	2910 \pm 623 (12)	<0.01
[³ H] fucose in urea/DTT resistant residue			
Days 1-5	2111 \pm 306 (8)	5866 \pm 756 (16)	<0.01
Days 7-21	4268 \pm 962 (8)	5074 \pm 682 (16)	>0.4
[³⁵ S] in ECTEOLA cellulose desorbate			
Days 3+5	911 \pm 262 (8)	2344 \pm 381 (15)	0.02
Days 7-21	1279 \pm 294 (14)	1551 \pm 294 (25)	>0.04
Days 1-2	1139 \pm 325 (8)	1472 \pm 297 (16)	>0.5
[³ H] proline in collagenase digest*			
Days 3+5 (tests)	3104 \pm 710 (16)	12,540 \pm 3727 (8)	<0.01
Days 1,2,11-21(tests)	3104 \pm 710 (16)	3747 \pm 795 (24)	>0.5
[³ H] proline in collagenase resistant residue*			
Days 2-3 (tests)	1800 \pm 263 (16)	4017 \pm 904 (8)	<0.01
Days 1,5-21 (tests)	1800 \pm 263 (16)	2205 \pm 330 (24)	>0.3

*controls were days 1-21 each comparison

TABLE 4a : RAW DATA OF TEST WOUNDS AND DERIVED RATIOS FOR FIGURE 4

DAY	[³ H] FUCOSE	[³ H] PROLINE	Na ₂ [³⁵ S] O ₄
1	161±60 (4) 4.13	211±28 (4) 1.31	369±112 (8) 1.5
2	292±94 (4) 5.67	734±233 (4) 1.7	153±20 (10) 1.6
3	170±60 (4) 5.88	1640±620 (4) 2.16	415±86 (8) 3.76
5	353.3±80 (4) 5.12	1952±957 (4) 4.11	147±52 (8) 4.88
7	161±37 (4) 2.93	1016±533 (4) 1.74	186±82 (7) 2.25
11	95±7 (4) 2.71	412±245 (4) 1.31	158±39 (8) 1.79
15	111±43 (4) 3.03	702±279 (4) 1.69	107±50 (6) 1.42
21	110±36 (3) 1.09	459±209 (4) 1.07	256±70 (4) 1.41

C.P.M. / mg FREEZE-DRIED WIEGHT ± S.E. (N) = REPLICATES

RATIO OF WOUND / CONTROL (BOLD FACE)

* Data reflect the day to day variation in incorporation. This was controlled for in that controls used were incubated at the same time as test wounds. However, this necessitated deriving the ratio of wound / control for comparison of different days.

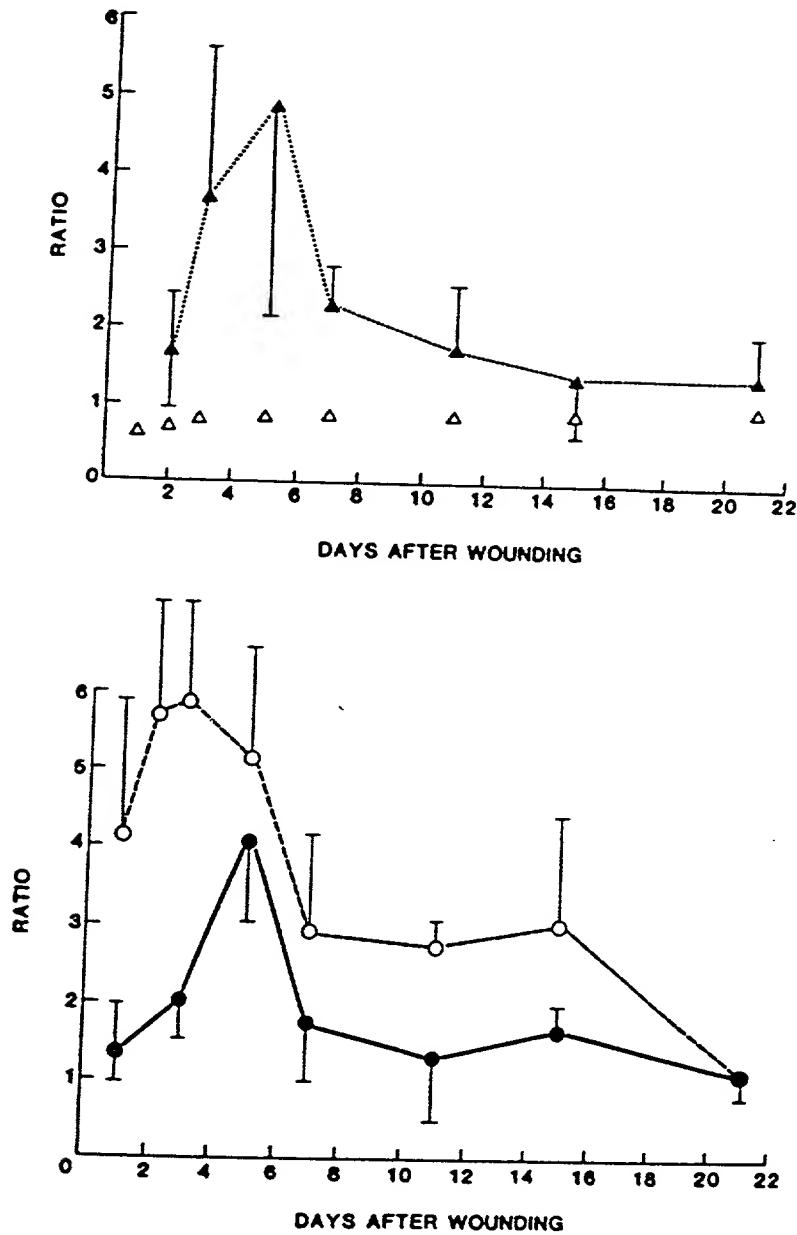


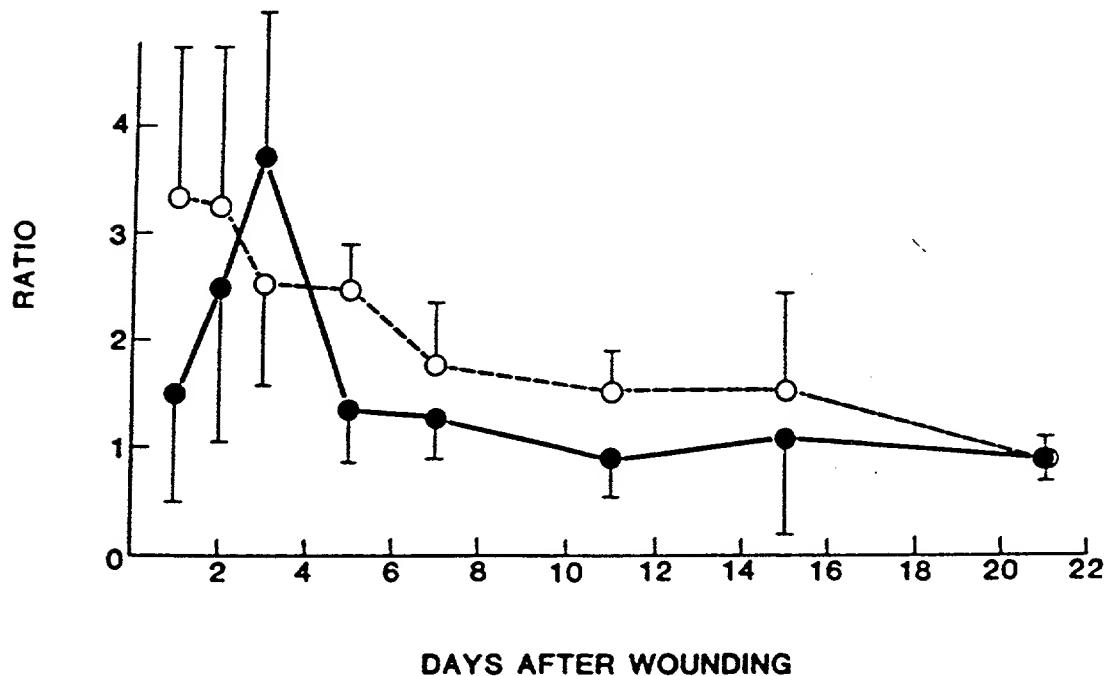
FIGURE 4

MEAN RATIOS (WOUND/CONTROL) FOR SOLUBILIZED SKIN FRACTIONS
 LEGENDS (SEE PAGE 63)

FIGURE 4 LEGENDS :

Radioactivity data were expressed as d.p.m./mg freeze-dried tissue homogenate before conversion to a ratio.

- [³H] fucose in urea/DTT extract (fucoprotein)
- ▲▲▲ [³⁵S] in ECTEOLA desorbate (glycosaminoglycan)
- [³H] proline in collagenase digest (collagen)
- △△△ weight of freeze dried homogenate

FIGURE 5
MEAN RATIOS (WOUND/CONTROL) RESIDUAL SKIN FRACTIONS

Radioactivity data were expressed as d.p.m./mg freeze-dried tissue homogenate before conversion to a ratio

- [³H] fucose in urea/DTT residue
- [³H] proline in collagenase resistant residue

incorporation group as the controls. If all the control discs which had been treated in the same way (though on different days) were pooled into one group (which forms the largest biological control population) then P values of <0.01 were found on comparing them with days 1-3 of the tests. Other comparisons with the control pool which were statistically significant at $P <0.01$ were days 3 + 5 for $[^3H]$ proline in collagen and days 2 + 3 for $[^3H]$ proline in collagenase resistant proteins and peptides. With the exception of $[^{35}S]$ in the ECTEOLA cellulose desorbate on days 3 + 5 and the $[^3H]$ fucose in the insoluble residue on days 1-5, no other test groupings differed significantly from the controls, either for the corresponding days, or for the whole time period.

DISCUSSION : The peak in specific activity of $[^3H]$ proline at day 5 in the collagenase digestible fraction confirms previous reports that the biosynthetic capacity of wound explants for collagen is greatest at this time 3,39,40. Of the other components studied, $[^{35}S]$ GAG biosynthetic capacity showed an early peak at the same time as collagen (fig 4). This is also in agreement with previous observations that a rise in certain of the sulfated GAG biosynthesis occurs 5 days after the production of a linear wound^{304,306}. The time sequence for the incorporation of fucose into the urea/DTT soluble glycoprotein components of the wounds differs significantly from proline incorporation into collagenase digestible components. The fucoprotein shows an earlier peak in biosynthetic capacity at 72 h after wounding (fig 4). $[^3H]$ proline incorporation in the collagenase resistant residue also peaks at day 3 (fig 5). Since

the incorporation of [³H] proline into the aminopropeptide of type III collagen would appear in the TCA precipitate from the collagenase resistant residue and since it would incorporate fucose as well¹⁸¹, the peak [³H] fucose incorporation on day 3 (fig 4) is likely contributed to by procollagen type III aminopropeptide synthesis. From this comparison it is also possible to infer that the peak for [³H] fucose incorporation into urea/DTT soluble material must be largely due to *de novo* protein biosynthesis of fucoprotein(s), since [³H] proline incorporation in the collagenase resistant residue correlates with the [³H] fucose incorporation in the urea/DTT soluble extract. Other salt soluble plasma glycoproteins have been reported to occur in wounds during the inflammatory phase²⁸, and it is possible that these also may act as acceptors for [³H] fucose³⁵⁴. It is unlikely that these represent the material in the urea/DTT soluble extract due to a reducible cross-linking of soluble plasma glycoproteins to an insoluble matrix, since the [³H] fucose labelled material is not extracted with 1.65 M NaCl. In contrast, material in the urea/DTT insoluble residue incorporates [³H] fucose maximally at 24 h when [³H] proline incorporation is low (fig 5). Fucosylation of this highly insoluble material by glucosyltransferases from plasma is possible³⁵⁴. The nature of this material is otherwise unclear except that it is not solubilized by boiling in saline, or reduction in 8 M urea³⁵⁵. It may be some as yet unidentified serum-derived fucoprotein, which is present early and is cross-linked to fibrin or itself.

Although previous reports agree there is an increased wound / control ratio for type III collagen, there is disagreement about the length of time this ratio stays high

after wounding and about the ratio of type III/type I collagen at various stages in the healing process. Gay *et al.*¹⁰² have shown that procollagen type III is present simultaneously with collagen type III and persists in wounds owing to a slower conversion to collagen compared to type I procollagen. Using an immunofluorescent technique, it was found that an increase in type III / type I collagen ratio persisted until 72 hours after implanting a sponge in human skin wounds¹⁰². Bailey *et al.*⁴⁰² found that the ratio of type III / type I collagen was increased for up to 21 days in the granulation tissue obtained by implanting a polyvinylchloride sponge in rat skin. However, using rat skin biopsy wounds as in the present work, Clore *et al.*³¹⁰ reported a wound / control ratio for type III collagen which was greater than 1 at 10 h post wounding, but returned to normal at 24 h. There is a discrepancy between these results and those reported earlier by Cohen *et al.*⁴⁰. The latter agree with the results presented in this thesis, that there is an increase in the wound / control ratio for total collagen biosynthesis at day 5. Clore *et al.*³¹⁰ found a normal type III / type I ratio and a wound /control ratio of 1 for type III collagen at day 5. This implies that the biosynthesis of both collagen types is not increased at day 5. This discrepancy may be accounted for by the pepsin-acetic acid extraction technique used by Clore *et al.*³¹⁰ which, as the authors suggest, may extract less total collagen, and proportionately less of type III, than the collagenase digestion technique used by Cohen *et al.*⁴⁰ and in the present work. The results in the present work are consistent with the hypothesis that there is an increase in type III procollagen biosynthesis three days after wounding.

followed by an increased type I collagen biosynthesis 5 days after wounding. The results do not provide any indication of how much type III and type I collagen remain in the wound as fibres. Conclusions from the present work also depend on the characterization of the [³H] fucose labelled material as type III aminopropeptide. This is addressed in section III , 5.

The continued elevation of the wound / control ratio for [³H] fucose incorporation during the period 7-21 days (fig 4) could be due to increased type III procollagen production if one accepts the view of Bailey *et al.* ⁴⁰², and Gay *et al.* ¹⁰² but not if one accepts the conclusions of Clore *et al.* ³¹⁰.

The foregoing study relies on the incorporation of [³H] into the glycoprotein fractions extracted from wound tissue incubations. In order to determine that the incorporation was that of authentic fucose in glycosidic linkage the following studies were carried out.

2. FUCOSE IDENTIFICATION IN THE WOUND GLYCOPROTEIN FRACTIONS

INTRODUCTION : The methods of fractionation adopted in the following work are modified from Timpl²⁸⁶. Incorporation of [³H] fucose into the various wound tissue fractions from explant culture was studied first for total recovery of radioactivity and then for recovery as [³H] fucose. To establish that the [³H] in the skin extracts was only in the form of [³H] fucose, it was necessary to hydrolyse the labelled extracts by methods which would only split off sugars and then to identify the labelled sugar(s) present. These techniques should show whether the [³H] fucose had been metabolized to other sugars, or whether non-specific [³H] exchange had taken place.

MATERIALS AND METHODS: The wounding and labelling procedures were based on those described above. Homogenization and extraction procedures were modified from those used to isolate laminin from glomerular basement membrane reported by Timpl²⁸⁶. 250 g male Sprague-Dawley rats were anaesthetized with Nembutal® i. p., and the dorsal skin was shaved. Wounds were made with a 3 mm dermal biopsy punch through panniculus carnosus. The animals were killed after 3 days and 4 mm discs of wounded skin were obtained with a dermal biopsy punch. Five tissue discs were incubated at 37° under 5% CO₂ : 95% O₂ for 6 h in 3 ml of Krebs-Ringer bicarbonate medium containing 150 µCi of L-[5,6-³H] fucose . The labelled discs were thawed 30 min at 37° and washed for 10 min in 10 mM fucose in 0.154 M NaCl. The tissue was homogenized at 4° in 6 ml of 15% glycerol (V/V) in 0.154 M NaCl, 50 mM Tris/HCl, ph. 7.5, containing 2 mM PMSF, 1 mM NEM, and 2 mM

EDTA using the PT-10 probe and the Polytron® homogenizer set at 8 for 60 sec and for a further 30 sec after sharply mincing with scissors any remaining large undivided skin fragments. The homogenate was tumbled on a rotator for 3 h at 20°, and then centrifuged for 30 min at 4° and 27,000 g (r_{av} 10.8) and the supernatant removed (S_1). The residue was rehomogenized with a glass stirring rod in 3 ml 3.4 M NaCl containing 2 mM PMSF, 2 mM EDTA, and 0.025% ϵ -amino caproate, sonicated for 15 sec and rotated overnight at 4° to extract remaining cellular and salt soluble protein. Centrifugation as for S_1 yielded supernatant S_2 and an insoluble residue which was resuspended in 3 ml 4 M urea containing 2 mM PMSF, and 1 mM NEM in order to extract uncross-linked fibrin and collagen. The suspension was rotated for 24 h at 20° and centrifuged as before giving supernatant S_3 . The insoluble residue was extracted twice with ethanol: ether (3 : 1 V/V) and finally with ether to remove lipid, and weighed after drying. The dried residue was suspended in water with sufficient dry chemical to make a 2 ml solution containing 8 M urea (Schwartz-Mann), 50 mM dithiothreitol, and 2 mM PMSF. After rotating for 48 h at 20° the homogenate was centrifuged at 4° and 12,000 g (r_{av} 10.8) for 10 min and the supernatant removed (S_4). For $[^3H]$ fucose identification, the S_1 and other extracts were dialysed against water and freeze-dried.

THIN-LAYER CHROMATOGRAPHY (TLC) OF S_1 HYDROLYSATE:
Monodimensional TLC was carried out using borate activated 250 μ Silica G / Reddi

plates (Fisher Scientific) as described by Lato *et al*³⁶⁵. Pooled S₁ samples from four animal preparations were hydrolysed with 1.0 N acetic acid for 10 h at 100° as described by Scicocca³⁶⁴. The hydrolysate was deionized on AG 501 double ion-exchange resin (Bio-Rad) columns which were washed with 8 column volumes of double distilled water and finally with 10 ml of 5% acetic acid. An aliquot of the final eluate was taken for calculation of radioactive recovery and the rest evaporated to dryness and stored at 4°.

Five sugar standards for comparison were chosen ; 2.5 µg each of xylose, mannose, glucose, fucose, and galactose were chromatographed as 1 µl of a stock solution diluted to 10 µl with methanol : water (1 : 1 V/V). This was either spotted manually or with the use of a Linomat applicator (Camag). Radioactive test samples resolved better with the latter technique, in which a 5-20 µl sample in methanol : H₂O containing ≈750 c.p.m. was applied to the origin as a 10 mm strip. A 100 pmole (2 µCi) sample of L-[5,6-³H] fucose (NEN), which had been subjected to the hydrolysis procedure alone or together with 100 pmole of each of the 4 sugar standards, containing ≈24,000 c.p.m. was spotted similarly and run as a radioactive standard.

The plates were 20 X 20 cm and samples were run in duplicate 1.5 cm lanes on either side. The plate was placed in a glass chromatography chamber at 22°, tilted nearly upright in the developing solvent system which was n-butanol-ethyl acetate-isopropanol-acetic acid-water (35:100:60:35:30). The solvent front was

allowed to run to the end of the plate but no further. The plate was removed and dried initially at room temp for 15 min and then at 110° for 1 h . One half of the plate or the desired lanes were then sprayed with a freshly prepared solution of 20 mg naphthoresorcinol in 10 ml ethanol and 0.2 ml of concentrated H₂SO₄. Color development occurred in 15 minutes at 110° and was stable for 18 h. R_f values were calculated from photocopies of the plates. The individual spots or entire lanes could be removed as 0.5 cm scrapings and counted as inhomogeneous samples in 10 ml of Biofluor (NEN) using a Searle Mark IV liquid scintillation counter .

TLC results are shown in table 5 and figs 6 and 7.

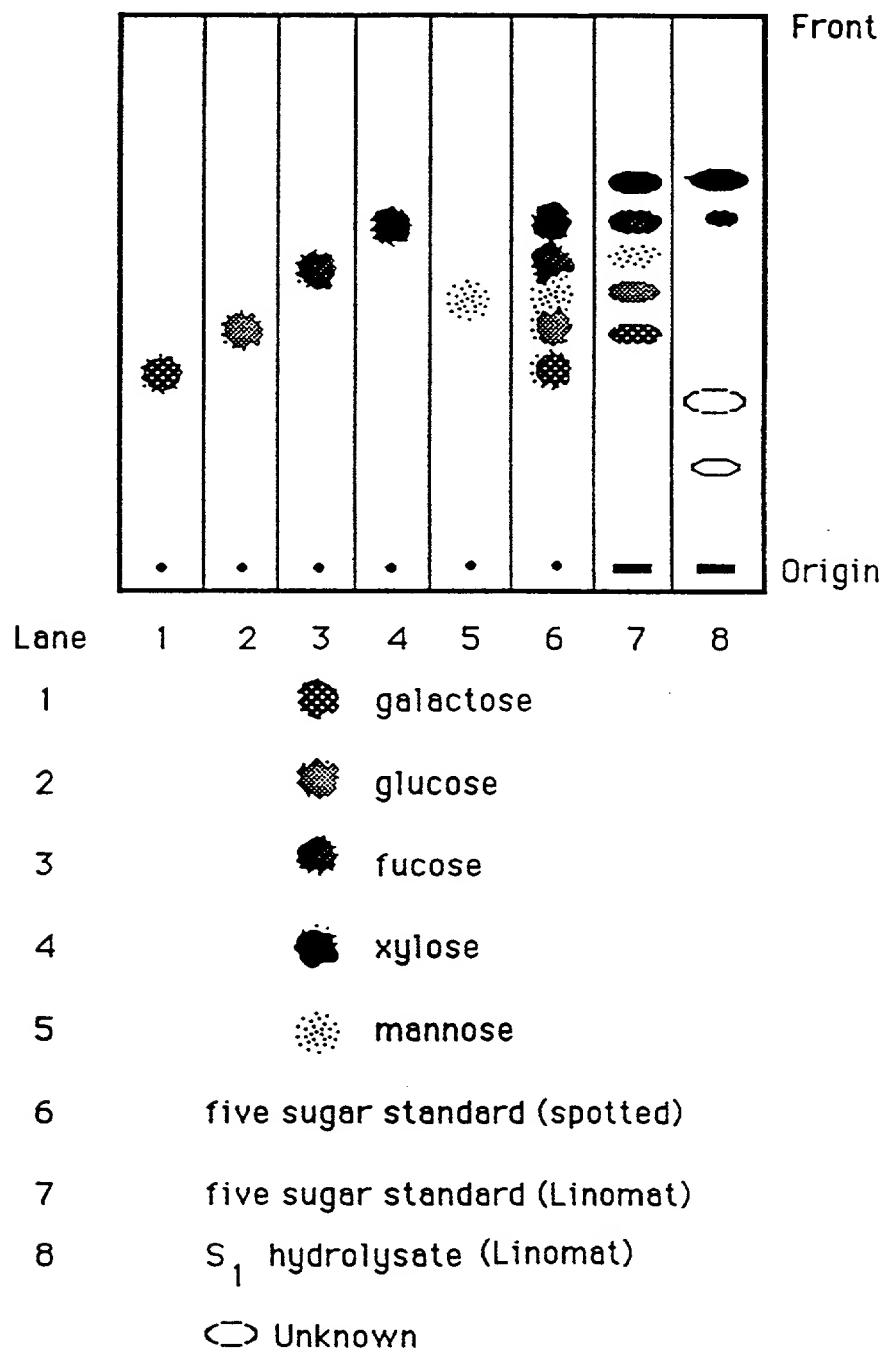
Table 5. Comparison of R_f values from TLC :

Linomat vs. manual spotting of 5 sugar standards*

Sugar	R_f Linomat	R_f manual spotting	Color developed
Xylose	68.4	66.7	blue-green
Fucose	64.7	60.9	purple-pink
Mannose	60.1	58.9	violet
Glucose	57.9	55.9	gray-violet
Galactose	52.6	50.4	gray

* R_f values a mean of three determinations

Figure 6. TLC of 5 sugar standards and S₁ hydrolysate
Conventional spotting and Linomat application



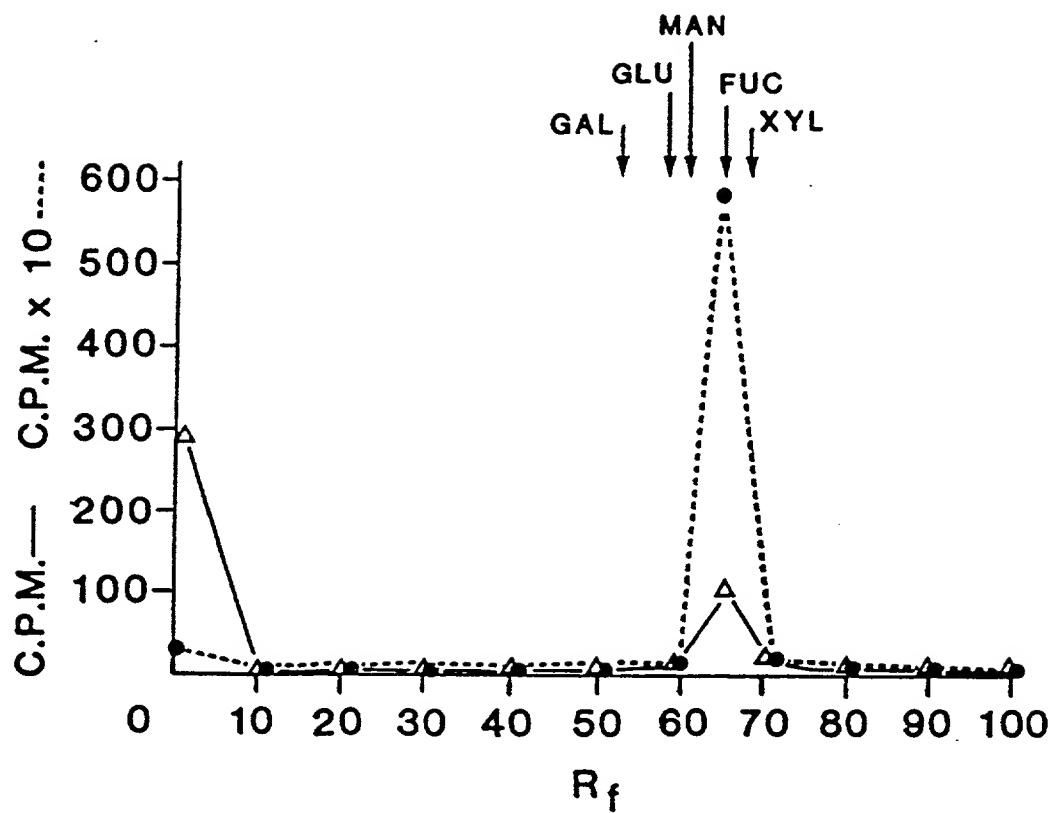


Figure 7. Silica Gel TLC: Acetic acid hydrolysates:

$[^{3}\text{H}]$ Fucose (authentic) with 4 sugars ●—●

$[^{3}\text{H}]$ Fucose S₁ hydrolysate pool △—△

RESULTS : A total of 30 separate 6 h incubations were carried out with [³H] fucose in the incubation medium. The average total incorporation was 90,000 c.p.m. in test wound incubations and 65,000 cpm in control skin. The average distribution in the various wound extracts is given in table 6. Over 50% of the non-dialysable [³H] fucose is incorporated in the S₁ fraction, with nearly 25% in the S₄ fraction. Approximately 1-2% was found in the organic and S₂ extracts and was not analysed further. S₃ extracts with 5.7% and insoluble residues with 15% made up the balance.

The results of TLC reveal that adequate separation of sugar standards is achieved with one dimensional analysis as seen in table 5. When a mixture of 4 sugar standards with [³H] fucose was subjected to hydrolysis conditions, the [³H] from fucose does not appear to exchange upon hydrolysis with the other 4 sugars since a single radioactive spot having an R_f = 64.8 was found (fig. 7). This corresponds with authentic fucose having an R_f = 64.7 (table 5). The labelled S₁ hydrolysate chromatographs as a single radioactive spot also having an R_f = 64.8 (fig. 7). A typical separation is depicted in fig 6.

Radioactive recovery from the S₁ hydrolysate on the ion exchange resin was nearly 100% in the 5% acetic acid eluate, with less than 0.1% appearing in the water washes.

DISCUSSION : Saline-glycerol (S₁) extracts of homogenized skin discs incubated with [³H] fucose for 6 h were found to contain greater than 50% of the total incorporated label (table 6). For this reason S₁ extracts were used to assess the fraction of incorporated label present as [³H] fucose. It was considered unlikely that the other extracts would contain different labelled compounds.

After acetic acid hydrolysis the final eluate of the deionized S₁ extract contained approximately 100% of the label. This indicated that the [³H] was in acetic acid-labile linkage rather than peptide linkage. TLC of the final eluate showed a single radioactive spot which corresponded in position with fucose, together with some labelled material which did not move from the origin (fig 7). The amount of this material decreased with increasing hydrolysis time. Therefore, it is presumed to be oligosaccharides. The naphthoresorcinol staining pattern and color development for the S₁ hydrolysate showed a small spot for fucose and xylose, but the identification of the other spots is not yet clear.

The presence of a fucosylated glycoprotein in wound extracts would be of little importance in the process of repair unless cross-linking to other structural components or itself could be demonstrated. It was on this basis that the following study of the effect of inhibitors of transglutaminase on the solubility of fucosylated wound glycoprotein fractions was carried out.

3. EFFECT OF POLYAMINES ON THE SOLUBILITY OF FUCOSYLATED WOUND GLYCOPROTEIN FRACTIONS

INTRODUCTION : The work described in Section III ,1 showed that a large fraction of the [³H] fucose-labelled glycoprotein produced by healing wounds is resistant to extraction with 1.65 M NaCl. It was speculated that this might be due to cross-linking with other components of the wound, possibly through the action of transglutaminase. Transglutaminases are a class of enzymes present in various tissues and specialized cells in the body which form isopeptide bonds between reactive glutaminyl groups of certain specific proteins and a variety of amines and amino groups, such as the ϵ - amino group of lysine (fig 3) ³⁷³. Plasma transglutaminase introduces ϵ -(γ -glutamyl)-lysine bonds between fibrin monomers, fibrin and collagen, and fibronectin and collagen, and may have an important role in wound healing ^{197,374,375}. Certain inhibitors of transglutaminase such as the polyamines bind to the acyl-enzyme intermediate as amine donors, resulting in the formation of analog-amine adducts ³⁷⁶. Putrescine (diaminobutane) is a naturally occurring aliphatic polyamine occurring in most living organisms ^{377,378}.

Putrescine was used as the putative polyamine inhibitor for several reasons. Firstly, incorporation of [¹⁴C] putrescine had been studied for various transglutaminases ³⁷¹. Secondly, it was incorporated by tissue transglutaminase but not plasma transglutaminase (factor XIII) into denatured type I and III collagen , co-polymerizing the latter with and without fibrin ³⁷² . It was suggested by Mosher ³⁷⁴

that transglutaminase may play a role in cross-linking collagen and fibrin during wound healing. Lastly, putrescine is not as toxic as other polyamines previously studied in the rat 370*.

An entirely different mechanism has been suggested for the effect of polyamines on the solubility of procollagen type II by Ryhänen *et al.* 367. It has been speculated that the effect is either due to direct inhibition of propeptidase, or to interference with the aggregation of procollagen molecules in the insoluble matrix, which results in higher concentration of soluble procollagen. The authors latter suggested that polyamines may play a useful role in controlling collagen accumulation in pathological fibrotic processes³⁴⁹.

It was the purpose of the experiments described in this section to examine the effect putrescine has on the concentration of insoluble fucoprotein in wound extracts. The mechanism of any effect will be examined in a later section.

MATERIALS AND METHODS : The incubations and extraction procedures have been described on pages 59 and 68. The effect of adding 50 mM putrescine HCl (Sigma) was studied on wound discs harvested on the third day post injury with regard to incorporation and distribution of [³H] fucose in the various extracts.

RESULTS : The results in table 6 compare the incorporated [³H] fucose in the non-dialysable fractions as a percentage of the total radioactive incorporation from 6 h incubations with [³H] fucose alone and [³H] fucose + 50 mM putrescine. The data are

* Registry Toxic Effects of Chemical Substances, 1-800-325-8070.

means \pm S.E. for 10 replicate incubations and subsequent extractions. Each [³H] fucose incubation was done at the same time as one incubation with [³H] fucose + putrescine and was paired with that one for the "t" test. Approximately 40% of the total incorporated radioactivity was found in the last two labelled fractions (S₄ + residue) in the absence of putrescine. In the presence of putrescine the percentage recovered in the insoluble fractions dropped to 32%, the difference being significant at $p < 0.02$. Conversely, the percentage increase in the soluble fraction (S₁) in the presence of putrescine is nearly equivalent to the decrement in the insoluble fractions. The difference between the S₁ groups is significant at $p < 0.01$. In these 6 h incubations a 30 % drop in total incorporation of [³H] fucose occurred in the presence of 50 mM putrescine.

DISCUSSION : Unlike previous data reported for 2 h incubations using other transglutaminase inhibitors, amino-acetonitrile (0.5 mM) or dansyl cadaverine (0.2 mM)³⁷⁰, there was a decrease in total [³H] fucose incorporation in 6 h incubations in the presence of 50 mM putrescine by 30%. However, the effect of this inhibitor on the ease of extractability of the material which incorporates [³H] fucose was clearly evident. That is, there is a significant increase in the percentage of non-dialysable [³H] fucose in the saline-soluble extract (S₁) in the presence of putrescine and a converse decrease in the percentage of [³H] fucose in the urea/DTT and insoluble residue. As shown later, the S₁ extract contains a single labelled species, a 14 kilodalton

TABLE 6

EFFECT OF INCUBATION WITH PUTRESCINE DURING [³H] FUCOSE LABELLING

EXTRACT OR FRACTION	% AGE OF TOTAL NON-DIALYSABLE [³ H] FUCOSE IN THE FRACTION OBTAINED FROM WOUNDS INCUBATED 6 H WITH :	
	[³ H] FUCOSE	[³ H] FUCOSE + PUTRESCINE
	Mean ± S.E.	Mean ± S.E.
SUPERNATANT ₁	53.6 ± 4.3	59.8 ± 3.4 ¹
3.4 M NaCl	1.2 ± 0.2	1.3 ± 0.2
4 M UREA	5.7 ± 1.2	5.0 ± 1.0
ORGANIC EXT.	1.0 ± 0.2	1.3 ± 0.3
8 M UREA/DTT	24.2 ± 3.8	20.3 ± 3.2 ²
RESIDUE	14.9 ± 1.9	12.0 ± 1.6 ²
TOTAL C.P.M. ± S.E.		
	97,385 ± 7772	76,417 ± 7742

¹ P < 0.01 FOR THE PAIRED "T" TEST OF THE FUCOSE VERSUS FUCOSE +
PUTRESCINE

² P < 0.02 FOR THE PAIRED "T" TEST OF THE FUCOSE VERSUS FUCOSE +
PUTRESCINE

fucoprotein (fig. 10). The increase in the relative proportion of [³H] in this extract in the presence of putrescine is evidently the result of less of the 14 kilodalton fucoprotein being retained or added to the insoluble pool. With this additional soluble material, resultant degradation and removal from the matrix is possibly increased, thereby decreasing the total non-dialysable incorporation. The length of the incubation may have accentuated a cell-cycle effect of putrescine on cell division and metabolism ³⁷⁷. The other explanation is that the increased proportion of the soluble fucoprotein may have inhibitory feedback effects on its production. Procollagen type I and III aminopropeptides have been reported to have such a self-regulatory role ¹²⁶. The direct effects of putrescine on accumulation of soluble glycoprotein could be due to altered processing of the molecule by carboxy- and aminopropeptidases as suggested above for procollagen type II. This is tenable for p-C-collagen which requires the C-terminal propeptide to remain soluble and would therefore accumulate in this form. However, the effect of putrescine on p-N-collagen may be to prevent conversion to collagen resulting in accumulation of the precursor due to steric or chemical interference with cross-linking ³⁴⁷. Notwithstanding, the effect of putrescine may have been as a result of inhibition of transglutaminase. This was studied further in wound culture by directing attention to transglutaminase mediated cross-linking of the 14 kilodalton glycoprotein.

4. TRANSGLUTAMINASE BINDS [3 H] PUTRESCINE TO 14 KILODALTON PROTEIN

INTRODUCTION : In view of the findings in table 6 it was considered possible that putrescine was inhibiting transglutaminase mediated cross-linking of fucoprotein.

The first step to prove this required labelling of S₁ and S₄ extracts with [3 H] putrescine and isolation of labelled γ -glutamyl putrescine ³⁷⁹.

MATERIALS AND METHODS : Tissue preparations were performed as described above with the exception that 50 μ Ci of [1,4 (n)- 3 H] putrescine (New England Nuclear) was used in the medium instead of [3 H] fucose. Extraction was carried out as described on pages 68-69.

HPLC OF PROTEOLYTIC DIGESTS : Freeze-dried S₄, S₃, and S₁ extracts containing 50,000 c.p.m from wound discs incubated with [3 H] putrescine for 6 h, were chromatographed on Sepharose CL-6B as described below. Portions of the effluent fractions were counted and fractions ranging from 0.54-0.58 K_D were pooled, dialysed exhaustively against distilled water and freeze-dried. The lyophilized material, containing \geq 20,000 c.p.m., was suspended in 1 ml of 0.2 M N-ethyl morpholine acetate buffer, pH 8.1, and subjected to sequential digestion with pronase, aminopeptidase M, and carboxypeptidase A and B, as described by Folk *et al.* ³⁸². Following digestion an equal volume of 10% TCA was added to the digest and the precipitate removed by centrifugation. The supernatant was extracted three times with ether and the aqueous layer was evaporated in an air stream until a 20 μ l volume contained \geq 1000 c.p.m. .

γ -glutamyl putrescine (generously provided by Dr J. E. Folk) was added to give a concentration of 2 nmol / 20 μ l . A 0.2 ml aliquot of the digest was mixed with conc. HCl to give a concentration of 6 N and was hydrolysed in a sealed tube at 105° for 24 h. The acid hydrolysate was evaporated to dryness over KOH and redissolved in 0.2 ml of water. The digests and the acid hydrolysates were each analysed on a 0.4 X 8 cm stainless steel column packed with Dionex DC-6A resin using a Beckman 322 HPLC pump system and the stepwise elution conditions described in figure 1 of Folk et al. 382. Fractions of 0.72 ml were collected with a Gilson 201 fraction collector, with elution time reported by Folk et al. 382 coinciding with fraction numbers in fig 16.

Fractions were analysed by counting 0.4 ml of each on a Beckman scintillation counter using HP/B scintillant .0.2 ml of each fraction was assayed in an Aminco-Bowman spectrophotofluorometer after derivatization with 0.2 ml of o-phthaldehyde reagent 383,404.

RESULTS : HPLC chromatography (fig 16) reveals the presence of γ -glutamyl putrescine as the major radioactive species accounting for 60 % of the total radioactivity recovered from protein hydrolysates, with the remainder appearing as a peak at the void volume which remains uncharacterized. After acid hydrolysis, 88 % of the radioactivity appears as putrescine alone, except for an unexplained peak in the peptide range. Several standards and replicates were chromatographed which reproduced the elution pattern in fig 16.

DISCUSSION : The fluorometric assay with o-phthaldehyde (OPA) was used to show the positions of the internal standard γ -glutamyl putrescine added to the proteolytic digest and putrescine derived from it by acid hydrolysis. The major radioactive peak found in the proteolytic digest of the 14 kilodalton peak from labelled S₄ extracts (fig 16) coincided exactly with the fluorescence peak for γ -glutamyl putrescine. Similarly, after acid hydrolysis the major radioactive peak corresponds with putrescine. The identification of γ -glutamyl putrescine shows that the 14 kilodalton material separated by chromatography has a transglutaminase substrate site. It shows as well that transglutaminase is present in the wound tissue discs. Whether or not the 14 kilodalton protein can be cross-linked to itself or to other proteins through the transglutaminase site is not shown by these experiments. In order to determine the nature of the cross-linked adducts produced by transglutaminase, studies were carried out to establish the distribution of fucose- and putrescine-labelled matrix components using column chromatography and SDS- polyacrylamide electrophoretic techniques.

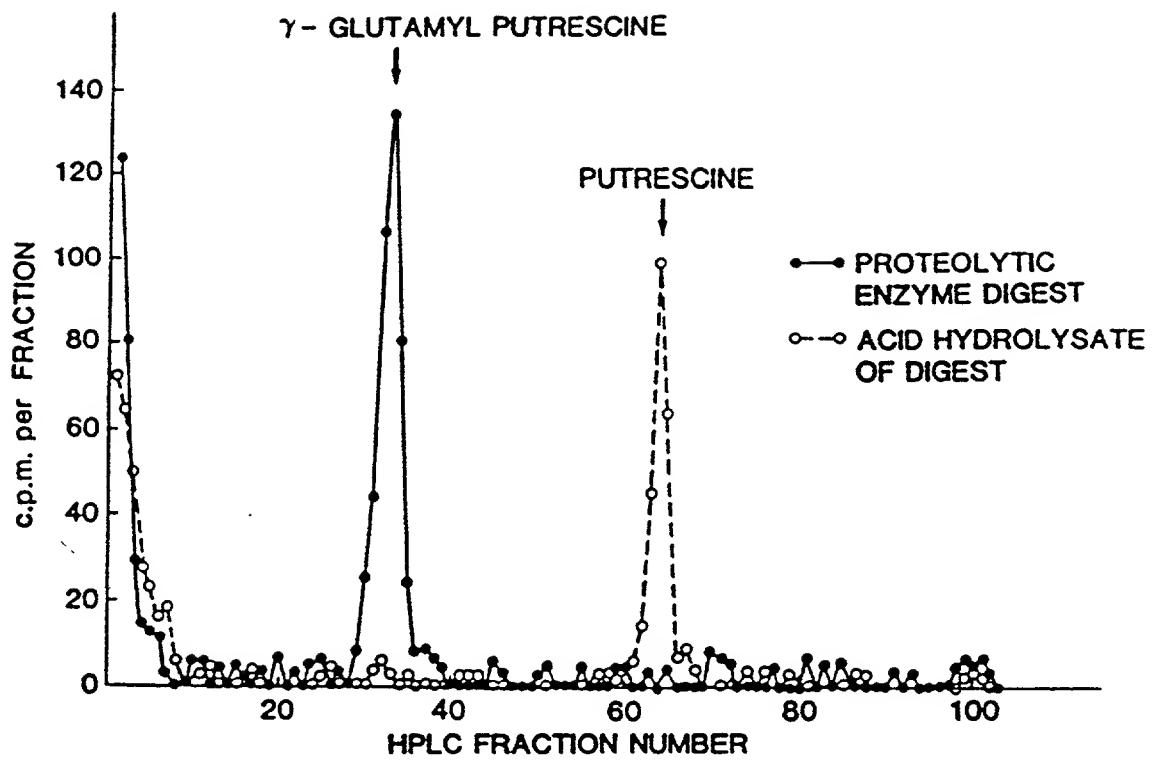


FIGURE 16. HPLC OF $[^3\text{H}]$ PUTRESCINE LABELLED S₄
(K_D 0.54-0.58 FRACTION)

5. CHARACTERIZATION OF MATERIAL LABELLED BY [³H] FUCOSE AND [³H] PUTRESCINE

INTRODUCTION : Having established that [³H] fucose and [³H] putrescine are incorporated in various fractions from wound explant culture, it was necessary to study the binding of one label with respect to the other. That is, would putrescine bind to the same protein(s) as fucose. In order to further characterize the labelled material gel chromatography was carried out using SDS / CL-6B Sepharose and repeated on peak fractions boiled in 8 M urea / 50 mM DTT. The column data were compared with that of pore-gradient electrophoresis of labelled fractions. The fucoproteins isolated by column separation were compared with each other by amino acid analysis.

MATERIALS AND METHODS : Parallel cultures were studied simultaneously from the same animals comparing [³H] fucose with [³H] putrescine incorporation. Extraction procedures were identical to those described on pages 55 and 69 with the following changes. The S₁ supernatant was dialysed twice for 48 h against 0.2 M NaCl in 0.1 M Tris/HCl pH 8.0 containing 1.0% SDS (TBS) with 2 mM PMSF. S₄ fractions were split with half being dialysed in TBS as above and the other half in 0.1% SDS (Bio-Rad) for subsequent concentration by freeze-drying . S₂ fractions were dialysed twice against 0.15 M NaCL in 0.01 M Na Barbital pH 8.0 (BBS). S₃ fractions were dialysed twice against BBS with 1.0% SDS. 6,000-8,000 molecular weight cut-off dialysis tubing was used throughout. Organic extracts were counted directly after

being filtered through 0.8 μ Millipore filters in Swinnex chambers. The filters were counted separately. Macromolecular incorporation of [3 H] fucose was determined for the control and test crude fractions and tabulated as % recovery of the total incorporation before being chromatographed.

For preparative separation on CL-6B Sepharose, a 55 cm long 1.6 cm diameter Bio-Rad column was used. Analytical chromatography was carried out with CL-6B Sepharose at 18° constant temperature on a 75 cm long 1.5 cm diameter LKB water-jacketed column, equipped with a Pharmacia 280 nm spectrophotometer and chart recorder. Samples were thawed if stored at -20° and sonicated for 15 sec.

Reduction and alkylation by boiling 2 min with 50 mM DTT and reacting for 30 min at room temperature with 0.1 M iodoacetamide in column buffer (TBS) was carried out in all cases.

A number of standard materials were also chromatographed at different times. 0.05 μ latex beads (Polysciences Inc., Warrington, Pa., USA) were used to determine V_0 , bromphenol blue was used for V_t and plasma fibronectin, γ -globulin, bovine serum albumin, ovalbumin, myoglobin, chymotrypsinogen, cytochrome c, ribonuclease A and lysozyme were used as molecular weight markers. These markers were used to prepare a straight line plot of log molecular weight versus K_D .

Column chromatography data were analysed for total [3 H] fucose recovery and specific activity of the effluent peaks in 10 ml of Aquasol II (New England Nuclear)

liquid scintillant after storage for 12 h in the dark at room temperature to reduce photoluminescence. Protein assay used was the technique described by Hess³⁶³ for solutions containing SDS.

Rechromatography of peak fractions from preparative gel filtration of 8 M urea/DTT extracts was carried out to determine the degree and nature of aggregate formation (figs 9 and 13). The samples were boiled for 2 min in 8 M urea / 50 mM DTT.

Amino acid analysis was carried out according to standard procedures on a modified Beckman LC Amino Acid Analyzer after acid hydrolysis of chromatographed freeze-dried peak fractions from S₄ extracts or from the S₁ peak fractions (figs 8 and 10).

ELECTROPHORESIS : Pore-gradient-SDS-electrophoresis was carried out according to the method of O'Connell and Brady³⁸⁰ with the following modifications. DHEBA cross-linker (Bio-Rad) 1.6% w/v in Tris/glycine buffer was mixed with 28.4 % W/V acrylamide stock solution (Bio-Rad) to give a 30% T : 5.3% C dense gradient solution. The light gradient solution was prepared from 9.6% W/V stock acrylamide and 0.45 % W/V DHEBA for a 9.6 % T : 4.7 % C solution. After addition of 200 μ l of 10% W/V electrophoresis grade SDS (Bio-Rad) equal volumes (22 ml) of each were filtered and degassed for 20 min at 20 °. 1.7 μ l TEMED (Kodak), diluted 1:10 with Tris / glycine buffer, was added to the dense solution , while 5 μ l of the TEMED solution was added to the light solution. 100 μ l of 25 % ammonium persulfate (Bio-Rad) was

added to each gradient solution immediately prior to pouring a linear gradient gel using the Hoeffer SE 600 slab gel system. 16 X 18 cm glass plates were used with 1.4 mm spacers. A pericardiocentesis needle was used with a Sorvall peristaltic pump to pour the gradient solution in from bottom upwards. The gels were underlaid with 25 % sucrose in Tris / glycine buffer and overlayed with butanol for 2 h at 4°. A pre-run of 2.5 h at 200 V : 20 mA constant voltage was carried out. Samples which had been concentrated by freeze-drying in 0.1 % W / V SDS were resuspended in an appropriate volume of sample preparation buffer containing 0.2 M DTT, sonicated 15 sec, and then boiled for 2 min. Approximately 100 µg of protein was applied to each well. Voltage was increased to 300 V : 30 mA and gels removed for fixing and staining within 10 h as described by Weber and Osborn ³⁸¹. After records of the gels were made, they were sliced on a grid and sample slices dissolved in 1 ml 0.025 M periodate for 48 h at 50°. Liquid scintillation counting was carried out in 10 ml of Biofluor (NEN).

RESULTS : Characterization of fucosylated material in 5 crude S₄ extracts was carried out initially on preparative CL-6B columns with a typical separation seen in fig 8. Radioactive recovery from columns averaged 85%. Three major radioactive peaks were identifiable with average K_D values of 0.55 (low molecular weight), 0.46 (middle molecular weight), and 0.42 (high molecular weight). The average specific activity (d.p.m./mg protein) of each peak was similar; 1165.7 d.p.m./mg, 793.1 d.p.m./mg, and 903.7 d.p.m./mg, respectively. Resolution of the peaks was improved

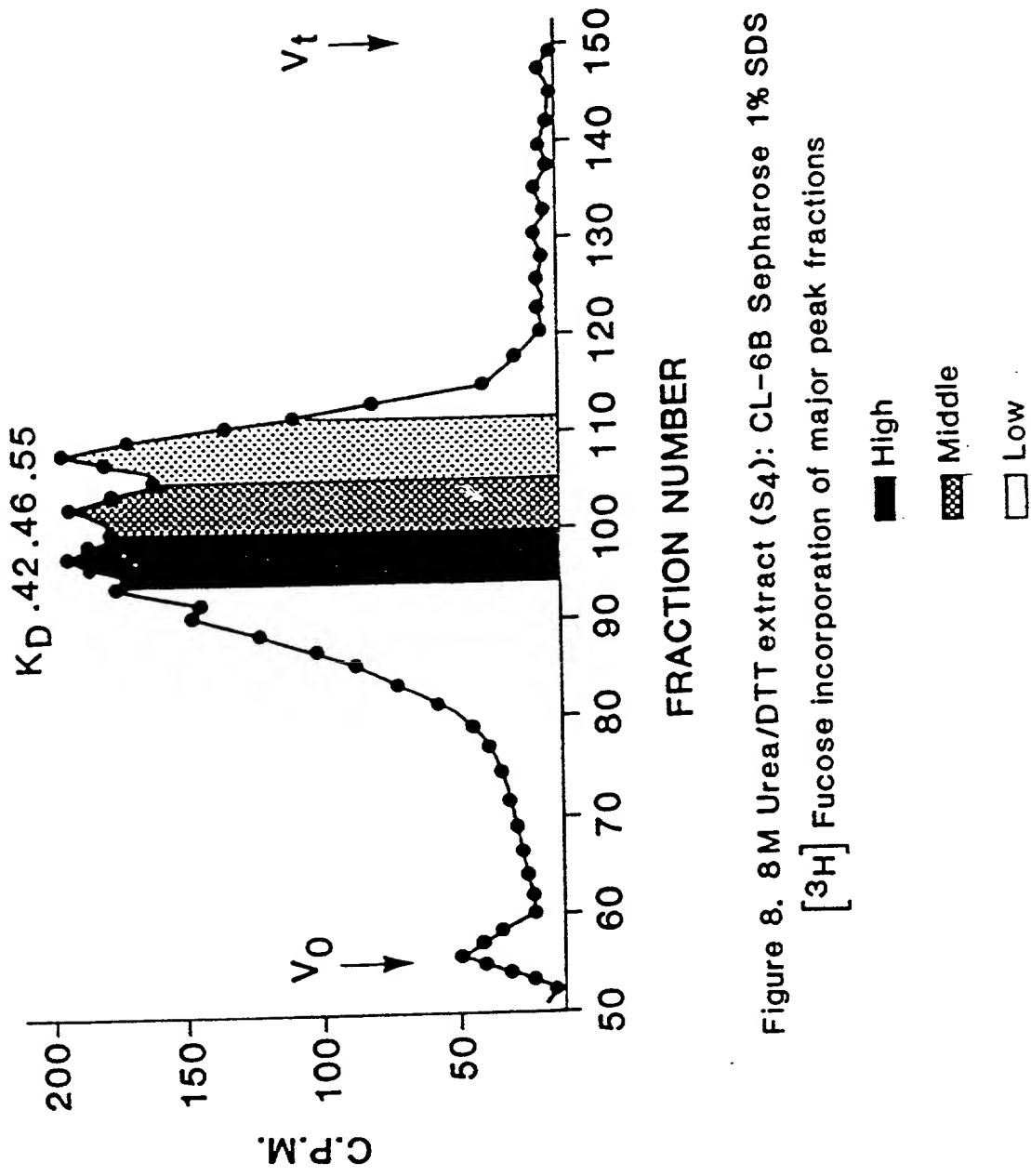
somewhat by taking the peak fractions of a pooled sample in 8M urea / 50 mM DTT and rechromatographing on the analytical column (fig 9). There was a corresponding increase in specific activity of the high molecular weight peak to 10,887.1 d.p.m./mg . A smaller increase was seen for the middle molecular weight peak to 2569.2 d.p.m./mg. The low molecular weight material at 2180 d.p.m./mg did not quite double. Accompanying this treatment a lower molecular weight peak appeared (K_D 0.59) with a lower specific activity (1500 dpm./mg) . In contrast, 5 separate S_1 extracts concentrated by precipitation at 4° with 80% ethanol prior to chromatography, eluted as single discrete fucosylated glycoprotein peaks on analytical columns with an average K_D of 0.55 (recovery 87%). Furthermore, the average specific activity of these peaks was 13,344.1 d.p.m./mg of protein (fig 10).

The 0.42 K_D material from S_4 extracts was compared with the 0.55 K_D material from S_1 extracts by amino acid analysis in table 7. Using the Difference Index of Metzger *et al.* the two are compositionally related with a D.I. of 6.6³⁶⁶. Samples were run in duplicate and results were averaged for comparison. Using log molecular weight versus K_D plots from standard proteins (data not shown) the molecular weight of the 0.42, 0.46, and the 0.55 K_D peaks are 43, 28, and 14 kilodaltons, respectively.

Coincident peaks at 0.55 K_D were seen in chromatograms of [³H]

fucose-labelled material and of [³H] putrescine-labelled material from S₁ and S₄ extracts (figs 11 and 12). The recovery of [³H] putrescine from columns compared favorably with that of [³H] fucose, being 90% on average. Furthermore, a similar relationship to that in fig 9 was evident when peak fractions from [³H] putrescine-labelled S₄ extracts were boiled in urea/DTT and rechromatographed (fig 13).

Glycoproteins are occasionally unusual in their behaviour in gel filtration³⁸³. Therefore, pore-gradient electrophoresis which is considered ideal for molecular weight estimates of glycoproteins was used to check the gel filtration findings³⁸⁴. Owing to the high concentration of protein necessary to achieve adequate counts in the bands, aggregation was seen to occur in the sample wells (figs 14 and 15). Notwithstanding, a protein staining band occurred in a M_r 14 X 10³ position in the S₁ extracts (lane C, [³H] fucose and lane D, [³H] putrescine), as well as in the S₄ extracts (lane G, [³H] fucose and lane H, [³H] putrescine). [³H] fucose and [³H] putrescine both appeared as radioactive bands containing 100 - 135 c.p.m. in the region of M_r 14 X 10³ as shown for S₄ extracts (fig 15). The absence of the two higher molecular weight peaks seen on CL-6B chromatograms of S₄ extracts is noted. Also noted is radioactivity which remains unentered at the top of the gel and at the front.



M.W. PEAKS RECHROMATOGRAPHED AFTER BOILING IN 8M UREA, 50MM DTT

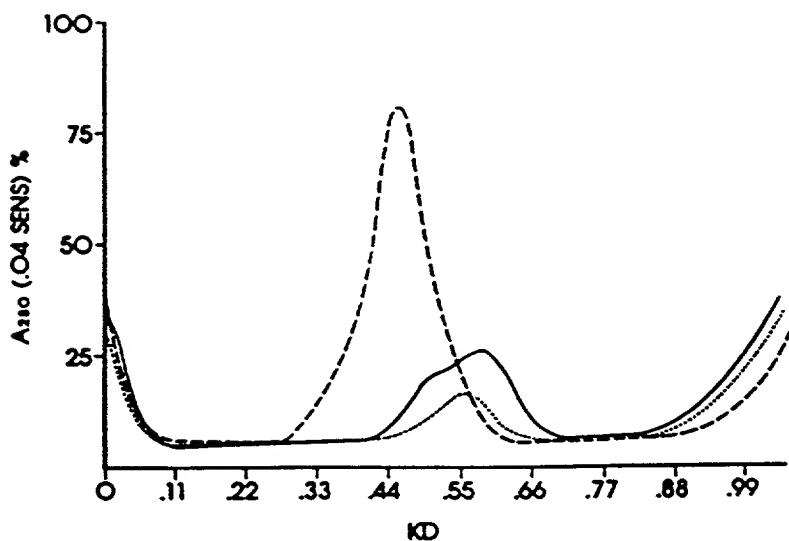
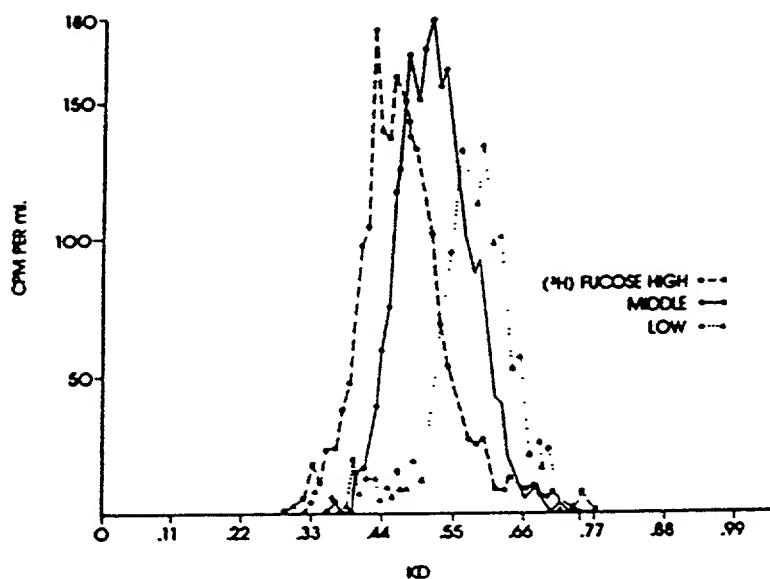


Figure 9. Rechromatography of peak fractions (S_4) after boiling in 8 M urea / DTT: CL-6B Sepharose :
 Top panel - C.P.M. / Fraction
 Lower panel - Absorbance 280 nm

LEGENDS TO FIGURE 9

[³H] FUCOSE
HIGH -----
MIDDLE ——
LOW
.....

Figure 9. Rechromatography of peak fractions (S_4) after boiling
in 8 M urea /DTT: CL-6B Sepharose

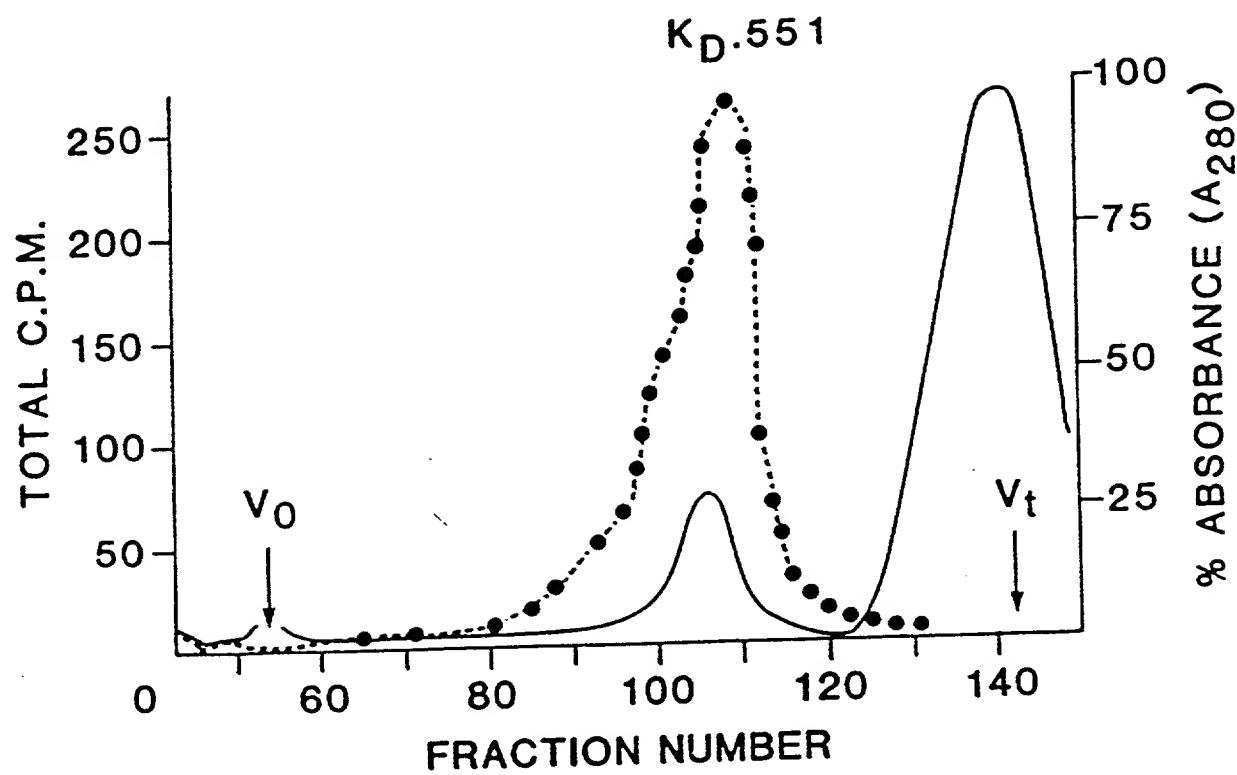


Figure 10. CL-6B Sepharose 1% SDS: $[^3\text{H}]$ fucose incorporation
 S_1 extract, C.P.M.----- Absorbance (A_{280})—

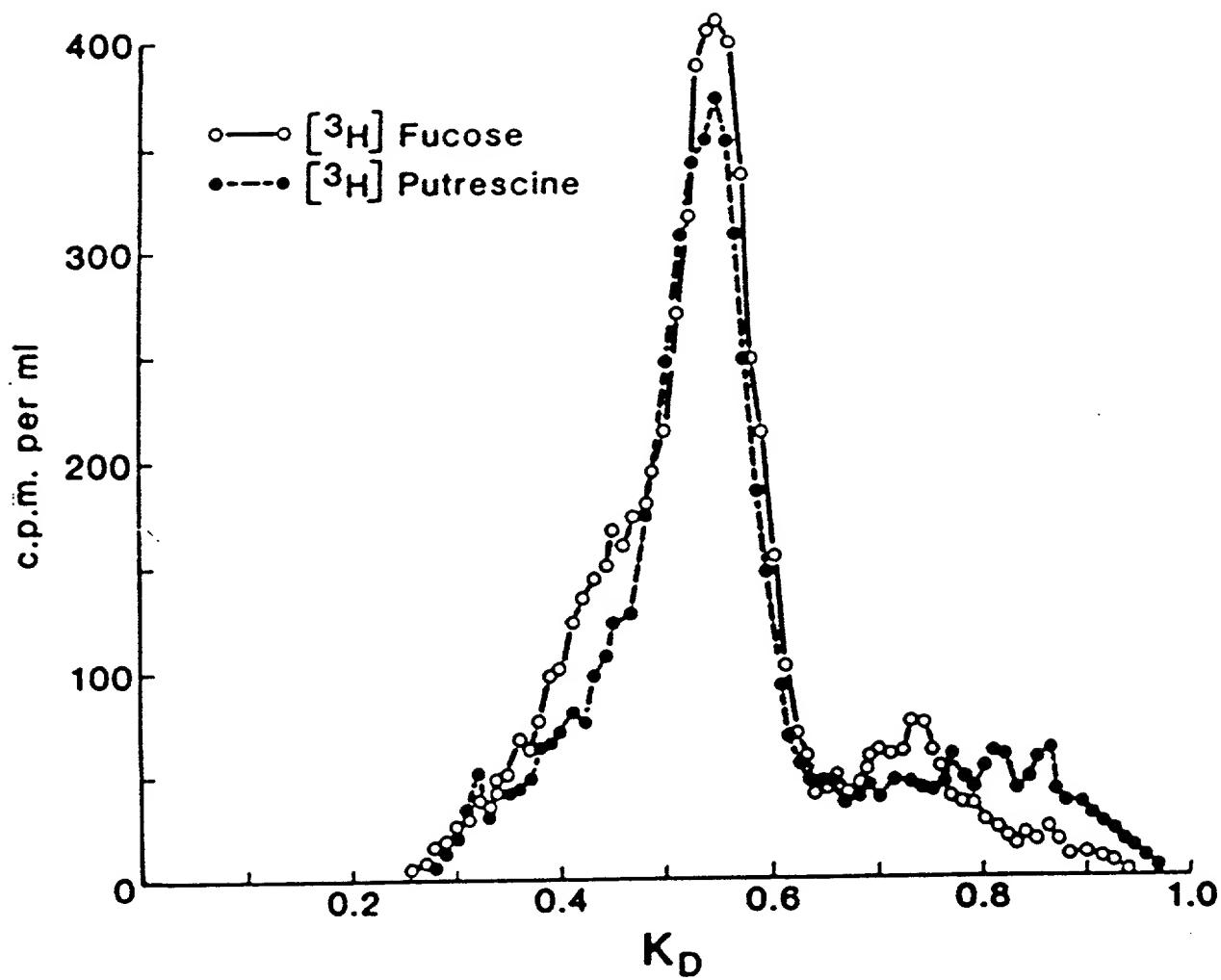


Figure 11. Chromatography in 1% SDS on CL-6B Sepharose of S_1

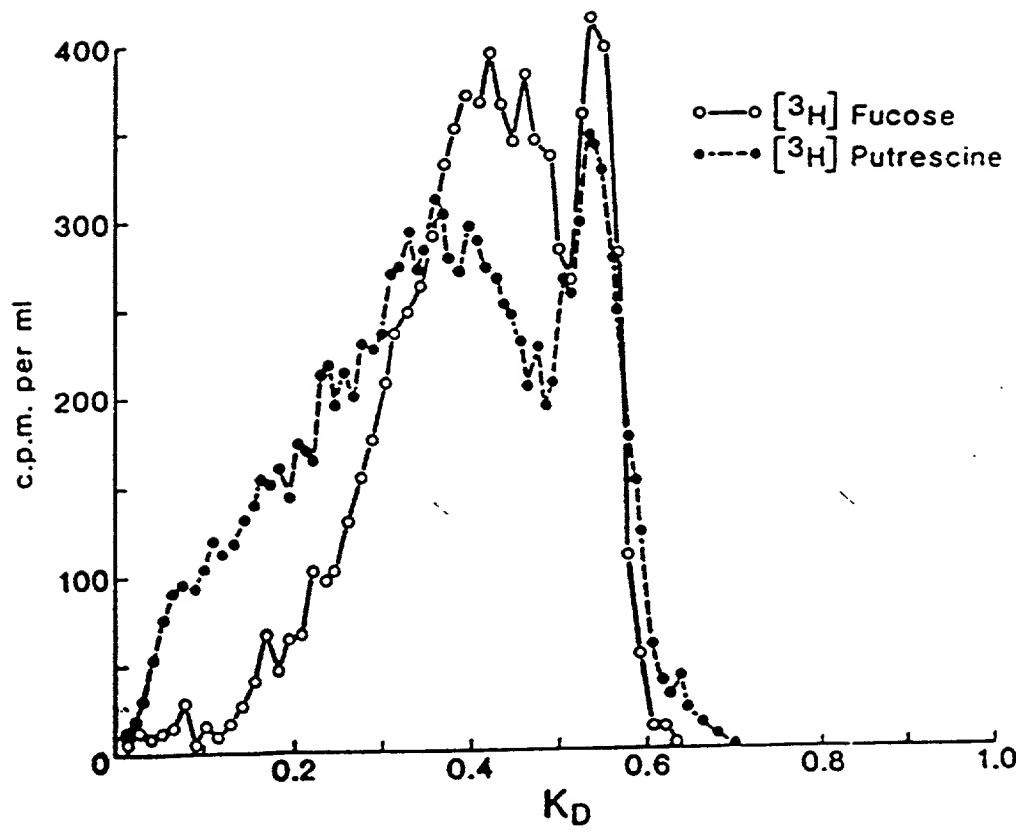


Figure 12. CL-6B Chromatography in 1% SDS of S₄ Extracts

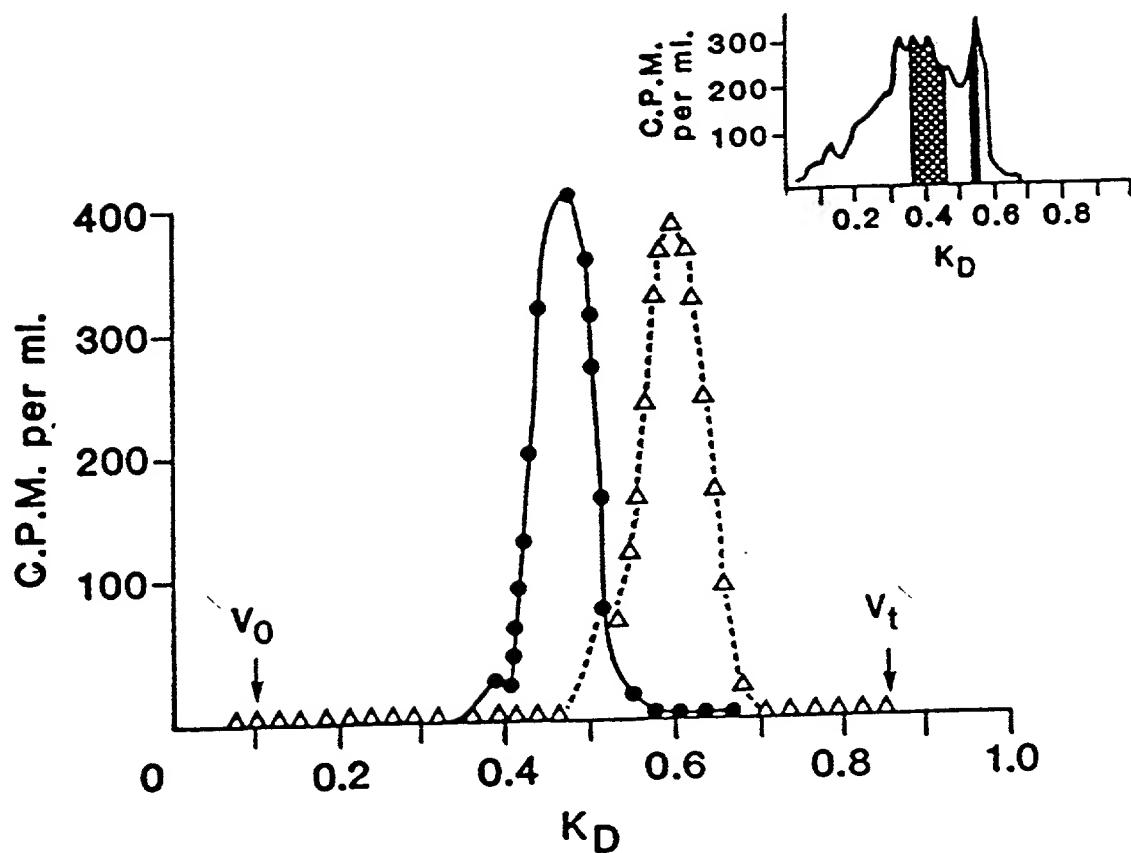


Figure 13. Rechromatography of S₄ Peak Fractions after Boiling in 8M Urea/ DTT: CL-6B Sepharose in 1% SDS

- [^3H] Putrescine Labelled High Range
- △-----△ [^3H] Putrescine Labelled Low Range

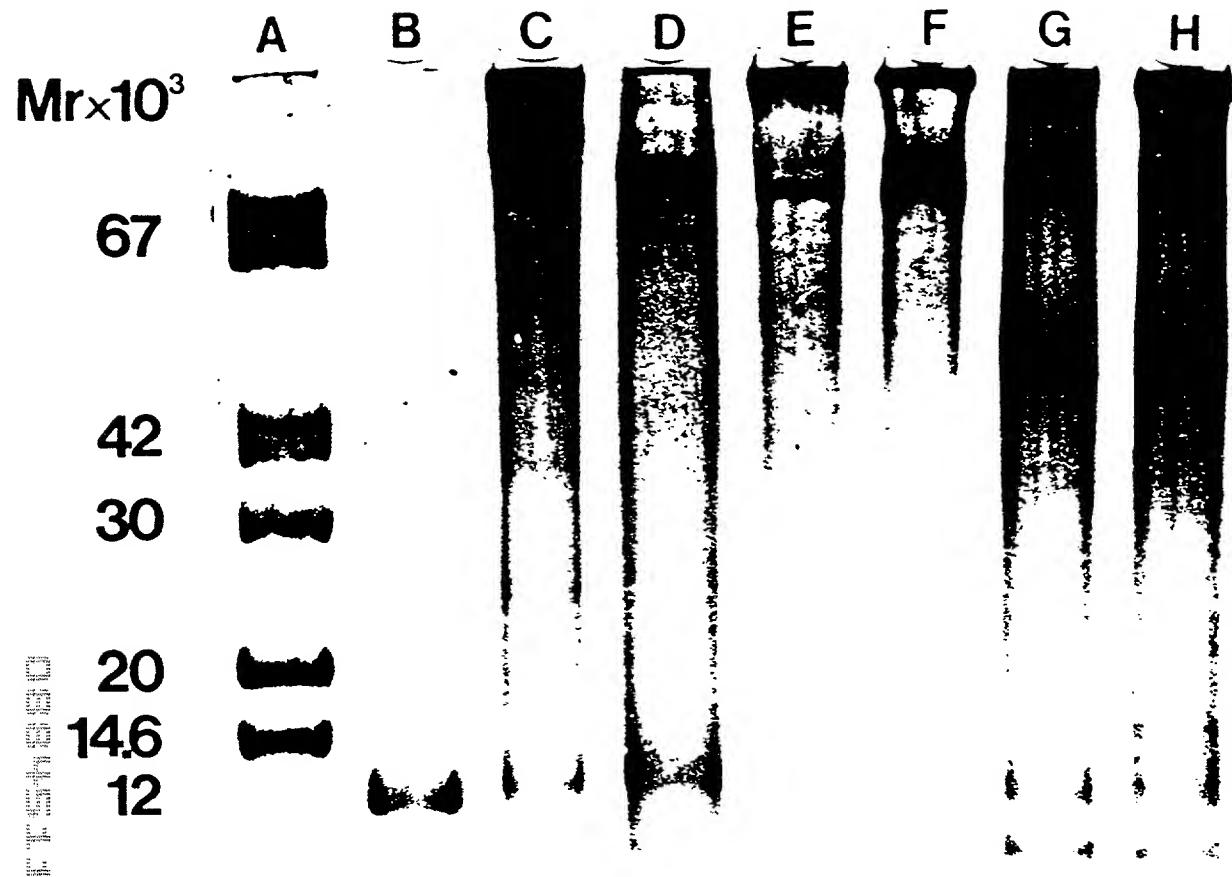


Figure 14 Pore-Gradient Electrophoresis :

S_1 S_3 and S_4 Extracts

Labelled with $[^3H]$ Fucose (Lanes C, E, and G) and

$[^3H]$ Putrescine (Lanes D, F, and H)

Standards (Lanes A and B): Bovine Serum Albumin, Ovalbumin,

Carbonic Anhydrase, Trypsin Inhibitor, Lysozyme, and Cytochrome C

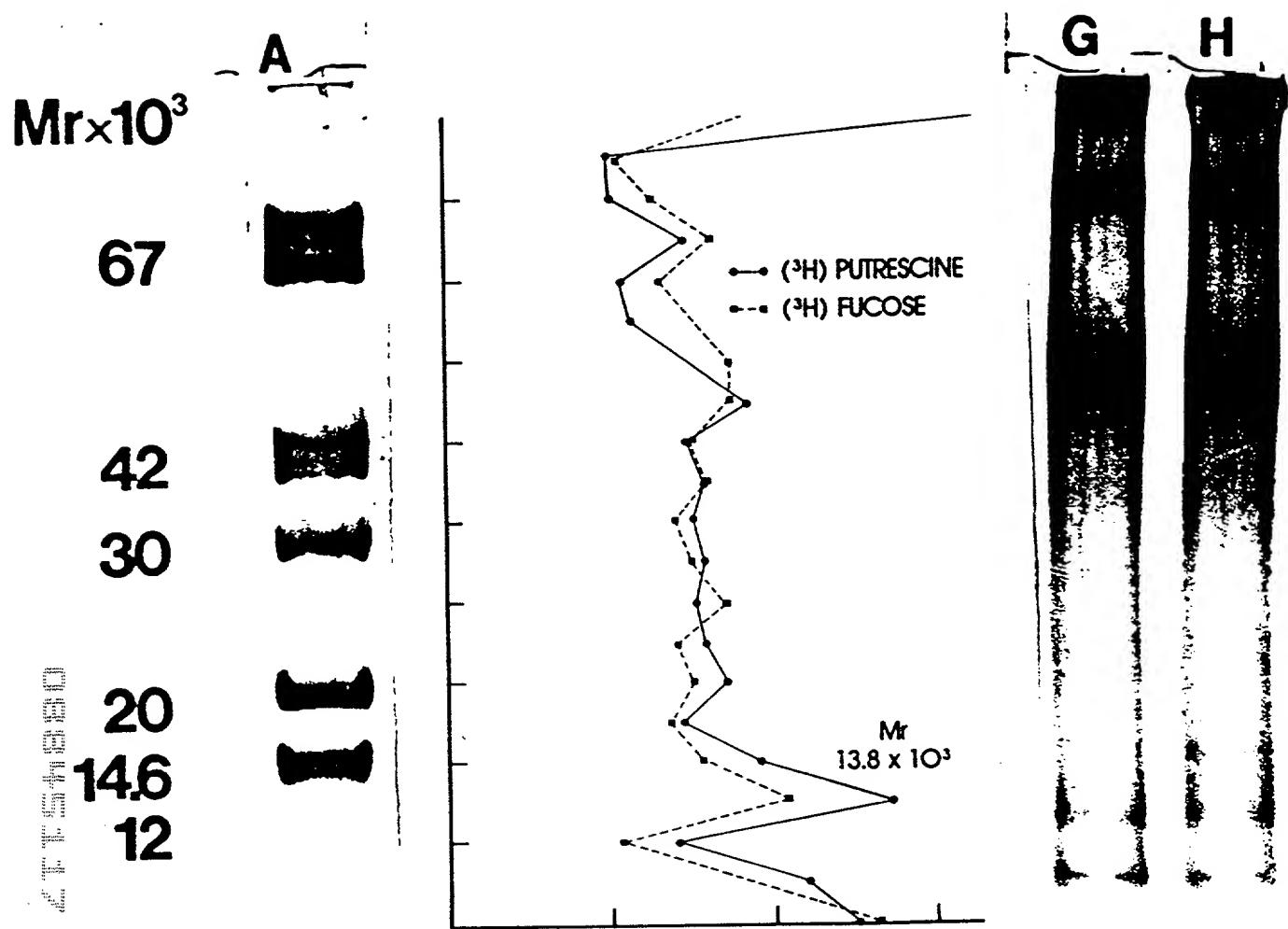


Figure 15. Pore-Gradient Electrophoresis of S₄ Extracts:

Gel slice * vs C. P. M. $\times 10^2$

- [³H] Putrescine
- [³H] Fucose

Standard and Sample Lanes as in Figure 14.

Table 7. Amino Acid Analyses: 42 and 14 Kilodalton Fucoprotein Peaks

Amino acid	Residues /1000	Residues /1000
Lysine	66.3	56.1
Histidine	18.2	21.5
Arginine	56.4	62.5
Aspartate	93.8	94.9
Threonine	50	45.3
Serine	63.8	73
Glutamate	85.5	100
Proline	55.1	63
Glycine	181.3	185
Alanine	72.9	81.9
Valine	51.2	24
Methionine	20.2	20
Isoleucine	40.9	32.3
Leucine	70.9	66.9
Tyrosine	25.5	12.7
Phenylalanine	28.7	18.6
Cysteic acid	16.9	N.D.
Difference Index:6.6.....	

6. GENERAL DISCUSSION :

The major fucosylated peaks found on chromatography of reduced and alkylated wound extracts were at K_D 0.42, 0.46., and 0.55. These are not likely the result of enzymatic degradation during the extraction procedure, since several protease inhibitors were used during each extraction and in the dialysis buffers. With the use of SDS in the column buffers it was observed that radioactive recovery from the columns was uniformly high. Washes performed with 1.0 N NaOH after several column runs confirmed this (data not shown). The size of the three peak fractions resemble the fucosylated glycoproteins described by Sear *et al.* ¹⁸¹ in human fibroblast culture.

Other glycoproteins have been reported in skin with approximately the same size as those described here, but there was no suggestion of a multimeric structural relationship or cross-link formation. Wolff *et al.* ³⁶⁸ found a number of high molecular weight bands in urea-mercaptoethanol soluble extracts of rat skin. These were mostly converted into a 15,000 M_r band by treatment with sodium borohydride. The authors stated that this might be due to either completion of the reducing process or to unspecific peptide cleavage. Randoux *et al.* ³⁶⁹ obtained two urea-mercaptoethanol soluble glycoproteins from rat dermis. Both had a molecular weight of about 16,000 daltons and both contained fucose. It seems possible that the material described in these earlier papers may be related to the reduced fucoprotein in the 0.55 K_D peak, which corresponds with a molecular weight of ~14 kilodaltons. Other early studies

indicated that dermal scar collagen contains a firmly bound glycoprotein not present in collagen from unwounded skin¹⁸⁸. Since wound tissue contains a greater proportion of fucosylated material extractable only with urea/DTT than unwounded skin discs, it seems likely that this fucosylated material is a component of the firmly bound glycoprotein associated with scar collagen³⁷⁰.

The nature of the firm binding of the 14 kilodalton fucoprotein may be seen as two-fold, involving disulfide cross-link formation as well as covalent cross-link production. This is suggested by the finding that a 14 kilodalton fraction is extracted by 8M urea / 50 mM DTT in the S₄ extract. Further reduction and boiling with urea fails to dissociate the higher molecular weight peak into smaller subunits (fig 9). It is possible that the high molecular weight fraction represents an unrelated fucoprotein which is coincidentally the appropriate size to be a trimer of a 14 kilodalton subunit. The likelihood of this seems doubtful on the basis of amino acid analysis (table 7). This was performed on a limited number of analyses of column fraction pools which had not been rigorously purified. However, the Difference Indices are in the same range as those comparing other purified proteins of known similarity³⁶⁶. Further purification of the peak fractions would be needed to compare their compositions definitively.

[³H] putrescine is known to label covalently a limited number of proteins which contain transglutaminase-sensitive sites³¹². The finding of γ -glutamyl putrescine in the digest of the 0.55 K_D putrescine-labelled peak (fig 16) provides strong evidence

that a 14 kilodalton protein subunit in wound granulation tissue has a transglutaminase substrate site, and that transglutaminase is present. Coincident labelling of the 14 kilodalton subunit by [³H] fucose and [³H] putrescine was shown to occur in two systems. Fig 11 shows that aqueous buffered saline-glycerol extracts (S₁) of wound tissue discs incubated with [³H] putrescine show a single radioactive peak when chromatographed on CL-6B in 1% SDS. This peak coincides exactly with the single peak found in extracts from incubations with [³H] fucose. The peak has a K_D value of 0.54-0.56, which corresponds to a molecular weight of about 14,000 daltons on a graph of K_D versus log molecular weight of standard proteins. When S₄ extracts were dialysed against 1% SDS and chromatographed in the presence of 1% SDS on CL-6B a 0.56 K_D peak again was evident after labelling with either [³H] fucose or [³H] putrescine (fig 12). As well, the two protein staining bands co-migrating with M_r 14 X 10³ in pore-gradient-SDS-electrophoresis labelled with both [³H] fucose and [³H] putrescine (figs 14 and 15).

Major column peaks were present at 0.36, 0.40, and 0.45 K_D that labelled with [³H] putrescine, which were not coincident with [³H] fucose-labelled peaks as seen in fig 8 or 12. A broad peak of protein was evident on the A₂₈₀ spectrophotometer recording throughout the higher molecular weight range and was labelled with putrescine to a lesser extent than the major peaks. This indicates that some of the

material labelled by [³H] putrescine is not the same as that labelled by [³H] fucose.

Whether the binding of putrescine resulted in a shift of multimeric fucoproteins into the monomer form by inhibiting covalent cross-linking was not studied rigorously.

However, the profile in fig 12 would support this possibility.

The main conclusion from the above findings is that rat wound tissue contains a 14 kilodalton protein subunit which has a transglutaminase substrate site and which corresponds in one chromatographic as well as one electrophoretic system with a 14 kilodalton fucoprotein. Further support for the hypothesis that it is the fucoprotein subunit which has the transglutaminase site comes from the use of putrescine on wound tissue *i n vitro*. It is known that putrescine in millimolar concentrations is an inhibitor of cross-linking by the enzyme³⁷³⁻³⁷⁹. Table 6 shows that 50 mM putrescine increases the fraction of non-dialysable [³H] fucose which is extracted from labelled wound tissue homogenate by saline-glycerol extraction (S₁) and decreases the remaining fraction, which is extracted by urea/DTT or is present in the residue. This indicates firstly that there is a fucoprotein in the extracts which is sensitive to transglutaminase inhibition by putrescine, and secondly that some of this fucoprotein is normally cross-linked through a transglutaminase site into the wound matrix.

A number of proteins which are substrates for transglutaminases or which bind [³H] putrescine are already known to occur in wounded skin. Epidermal keratins, which contain ϵ -(γ -glutamyl) lysine cross-links formed by transglutaminase give rise to a

number of subunits after treatment with 8 M urea and a reducing agent³⁸⁵⁻³⁸⁷. The number of subunits is controversial, but none contains fucose or has a molecular weight less than 49,000 daltons. A substrate for epidermal transglutaminase has been isolated from bovine snout³⁸⁸. The 36,000 dalton complex dissociates into subunits of 8-10 X 10³ daltons in 8 M urea or 1% SDS and it is found not to contain carbohydrate.

Transglutaminase is well known to catalyse fibrin cross-linking during blood coagulation³⁸⁹. Fibrin does not contain fucose and the molecular weights of individual fibrinogen chains are 63.5 (α), 56 (β), and 47 kilodaltons (γ)^{390,391,389}.

The 0.56 K_D peak (14 kilodalton) labelled with either [³H] fucose or [³H] putrescine (fig 12) does not coincide with any of these previously described protein subunits known to bind [³H] putrescine. One or more of the other [³H] putrescine labelled peaks (fig 12), however, may correspond with keratin or fibrinogen subunits.

The *in vitro* incorporation of [³H] fucose into a urea/DTT extracted fraction of skin wound discs reaches a peak 72 h after wounding (fig 4), which is well before the peak synthetic capacity of the wound for total collagen or a form of GAG sulfate polymer. The incorporation of [³H] fucose into the residue remaining after urea/DTT extraction is at its maximum 1 day after wounding and declines thereafter (fig 5). These findings are consistent with the hypothesis that biosynthesis of fucoprotein increases for three days after wounding, but cross-linking is at a maximum within 24 h of wounding. As discussed in section II-1, the temporal relationship of fucoprotein synthesis with that

found by Gay *et al.* and Bailey *et al.*^{102,402} for collagen type III would be explained if they were being produced from the same procollagen type III pool. Other explanations are possible and further work is required to distinguish these.

It is now known that the aminopropeptide of type III collagen, which consists of 15 kilodalton subunits linked by disulfide bonds, is an avid substrate for liver transglutaminase (Bowness, Folk, and Timpl⁴⁰⁹). Characterization of procollagen type III aminopropeptide from rat skin has not been carried out. Nevertheless, bovine type III aminopropeptide and the 14 kilodalton fucoprotein are structurally similar³⁹³. It seems likely that the collagen type III aminopropeptide subunit comprises a significant portion of the [³H] fucose- and [³H] putrescine-labelled material in the skin wound extracts but it is not established at present if other similar proteins are present.

From the above experimental work a theoretical model can be developed to explain the role of transglutaminase in the wound matrix and provide a unified mechanism of collagen fibre formation. It was shown in fig 1 that fibrin strands in the wound form an early matrix upon which fibroblasts migrate into the wound on day 3. The glycoprotein component present as 14 kilodalton fucoprotein in the wound on day three, as well, is available to be cross-linked. As has been suggested by Mosher³⁷⁴ fibrin and collagen may be cross-linked by tissue transglutaminase(s). This would allow the fucoprotein and collagen type III to be immediately incorporated into the insoluble fibrin matrix. Should the identity of the 14 kilodalton fucoprotein be established as the aminopropeptide of type III collagen, then the rate of fibrillogenesis

and orientation of interstitial collagen type III in the connective tissue matrix would be under possible control by transglutaminase. This could be due to enhanced triple helix formation of procollagen type III molecules before the aminopropeptide is removed by aminopropeptidase. Triple helical collagen type III present in the insoluble matrix is possibly the structural nucleus with which collagen type I can then form hybrid fibrils, which eventually give rise to stable mature fibres with the ongoing synthesis of collagen type I and subsequent diminution in the relative amount of collagen type III. The potential use of such an enzymatic control mechanism for collagen cross-linking during early wound healing would be to decrease the amount of procollagen III

cross-linked and available for hybrid formation. In pathological scars an excess of collagen type III has been demonstrated by Weber *et al.*⁴⁰⁸. Testing various transglutaminase inhibitors in the model should be possible by measuring the amount of type III collagen in early wounds, or by determining a decrease in the wound strength early in the process which is regained once collagen type I synthesis is eventually established. The effect of transglutaminase inhibition on wound healing using topical putrescine *in vivo* has been presented by the author since completion of the data in this thesis⁴⁰⁵. This preliminary report indicates that during the early phases of wound healing, both histologic and tensiometric data support a role for transglutaminase in wound matrix repair. The transglutaminase sensitive site on the aminopropeptide of collagen type III has recently been identified by Bowness, Folk, and Timpl⁴⁰⁹. This supports the model proposed above as the mechanism of early wound matrix assembly and stabilization by transglutaminase through the aminopropeptide of collagen type III.

7. SUMMARY OF CONCLUSIONS

A fucoprotein is found associated with skin and skin wounds. It is maximally synthesized in rat granulation tissue by day 3 post injury and declines in the third week to control levels. Most of the newly synthesized fucoprotein is extracted with saline-glycerol buffers and after reduction, chromatographs in 1% SDS as a 14 kilodalton peak on Sepharose CL-6B. However, additional 14 kilodalton fucoprotein is extractable from the wound matrix with chaotropic agents under reducing conditions, which implies that some is firmly bound in the connective tissue matrix by disulfide cross-links. Fifty mM putrescine renders the insoluble fucoprotein fraction increasingly saline-soluble. This suggests that a portion of the 14 kilodalton fucoprotein is bound in the matrix by transglutaminase-derived isopeptide bonds. $[^3\text{H}]$ putrescine also labels a 14 kilodalton protein when incubated with skin wounds *in vitro*. The $[^3\text{H}]$ putrescine was found to be bound to the 14 kilodalton protein as γ -glutamyl putrescine confirming a transglutaminase substrate site. The reduced and alkylated fucoprotein co-migrates in two systems with a 14 kilodalton $[^3\text{H}]$ putrescine-labelled protein. Although the fucoprotein has not been identified in this work, it was found to have some characteristics in common with the aminopropeptide of type III collagen.

It is hypothesized that isopeptide cross-linking of the fucoprotein may be necessary for growth of collagen fibres in the wound tissue matrix. Therefore, use of transglutaminase inhibitors may be of therapeutic consideration in disorders of fibrous proliferation such as hypertrophic scar and other fibrocontractive disease processes.

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